"THE APPLICATION OF COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY TO COMPOUNDS OF BIOLOGICAL INTEREST"

Subtitled

"STEROID ANALYSIS BY THE USE OF GLASS OPEN TUBULAR GAS CHROMATOGRAPHIC COLUMNS"

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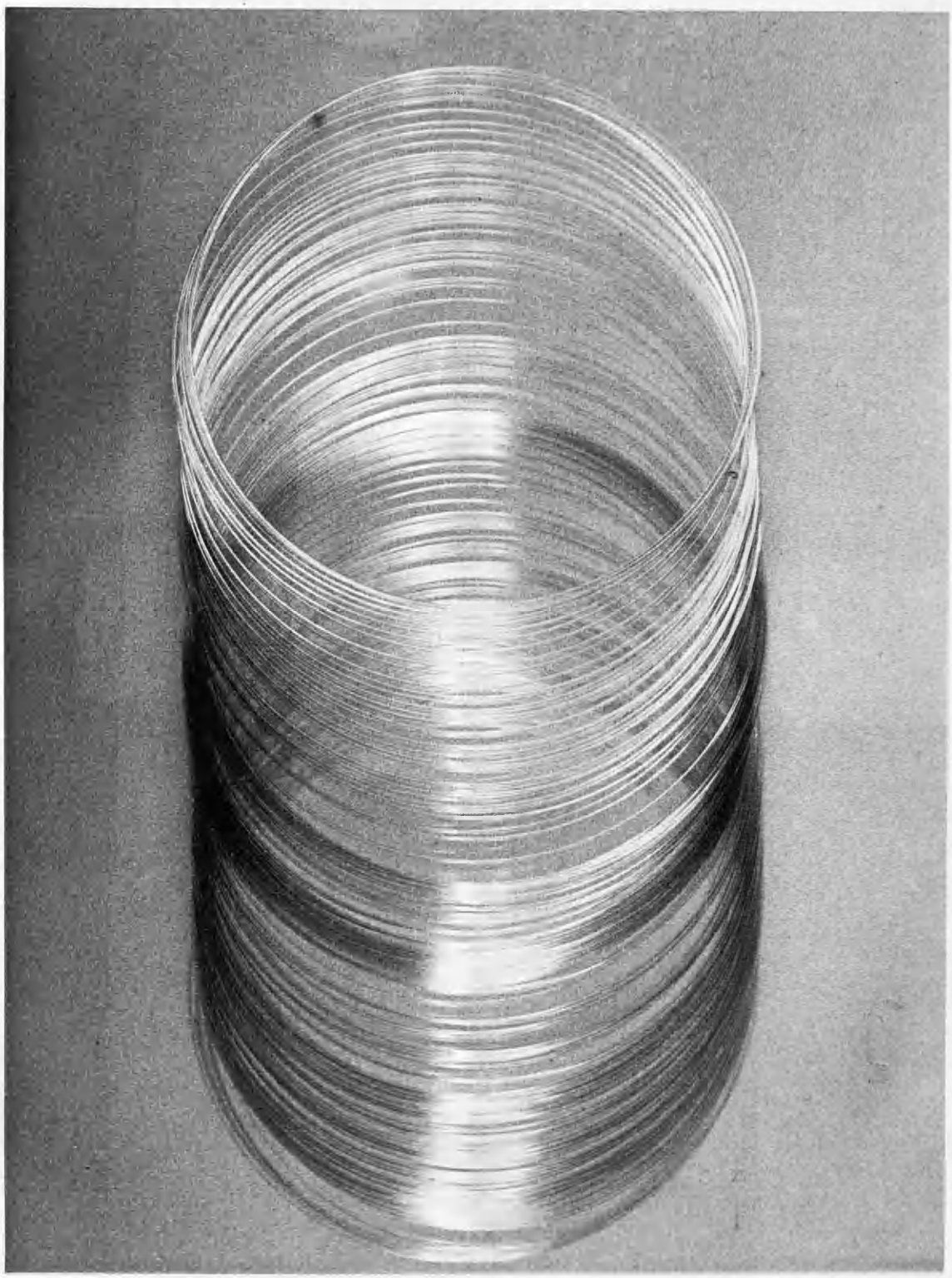

Frontispiece. 0.5mm x 60m uncoated open tubular glass spiral

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Summary

Steroidal compounds are virtually ubiquitous in nature and frequently occur in complex mixtures as constituents of closely similar structure. Packed column gas chromatography-mass spectrometry (g.c.-m.s.) is an analytical technique of unique facility for the simultaneous separation and characterisation of complex mixtures of organic compounds. This thesis examines the additional facility provided by glass open tubular gas chromatographic columns of higher efficiency in the analysis by g.c. and g.c.-m.s. of complex mixtures of steroidal compounds derived from a number of natural sources.

A method for the preparation of stable, efficient and reproducible glass open tubular gas chromatographic columns is described. The construction of chromatographic systems adapted to their somewhat more critical requirements is outlined. Particular attention is paid to the requirements for the interface of these columns to an LKB 9000 combined gas chromatograph-mass spectrometer. The performance of this system with emphasis on the nature of the advantage obtained over conventional packed columns is demonstrated by several model separations of mixtures of closely related standard steroidal compounds.

Mixtures of sterols derived from yeast and marine sources frequently consist in mixtures of components differing in the degree and position of alkylation and unsaturation in the basic cholestanol structure. Correlation of gas chromatographic retention, on glass open tubular columns of OV-1 stationary phase, with sterol structure is described. Complementary data available in the literature are integrated into a scheme for the rationalisation of increments of Kováts retention index associated with particular alterations in sterol structure. This system and mass spectral correlations obtained by glass open tubular g.c.-m.s. is applied to the analysis of sterol mixtures derived from five species of marine invertebrate and two mutant strains of the yeast Candida albicans. Two other applications to sterol analysis are also described. A significant advantage is demonstrated over g.c. and g.c.-m.s. methods heretofore employed.

Mixtures of hydroxy and ketosteroids may be derivatised as the alkyloxime-trimethylsilyl ether derivatives for g.c. and g.c.-m.s. analysis. The occurrence of syn- and anti-isomers in the alkyloximes of various ketosteroid structures is a complicating factor in their

g.c. analysis, in particular at higher column efficiencies. Several alkyloximes of increasing bulk of the O-alkyl substituent were examined in this respect. The methyloxime is shown to provide the least complications for open tubular g.c., though the "group separations" of hydroxy and ketosteroids provided by the higher alkyloximes may provide useful correlations. The utility of this approach is demonstrated in the analysis of mixtures of standard hydroxy and ketosteroids. Preliminary results obtained on a mixture of urinary steroid hormone metabolites of the human newborn by open tubular g.c.-m.s. of the isopentyloxime-trimethylsilyl ether derivatives are reported.

1. INTRODUCTION

1.1. General Introduction

Analytical chemistry has as its object the identification and quantification of the chemical constituents of sample materials. The nature and complexity of these samples may vary dramatically and their analysis covers the whole range of scientific inquiry. The validity of any method employed for these analyses may be assessed on the following criteria:

-accuracy: the agreement between the measured and actual amounts of substance analysed,

-precision: the agreement between replicate determinations of the same analysis, a broad index of the reliability of the method,

-sensitivity: the smallest amount of material which can reliably be measured,

-selectivity: the extent to which the method measures only the substance in question.

In addition to the reliability evaluated by these points, simplicity, speed and cost must be considered. In recent years many of the classical techniques have been superseded by physical methods of analysis. These instrumental techniques have, in most cases, proved superior on all points with perhaps some concessions on the criterion of cost.

Classical techniques relied on the comparison of physical and chemical properties for identification. A structure determination was often a complex series of operations which culminated in the synthesis of materials for direct comparison with the 'unknown'. The reliability of such comparisons depends on the purity of the substances involved. In reality the 'unknown' is rarely available in pure form but more often in mixtures of varying complexity and sometimes in limited quantities. Before the advent of g.c.-m.s. in 1957 it was impractical to consider the complete analysis of mixtures consisting of 20 unknown components or more and impossible if the quantity available for analysis were not of the order of grams. Today, identification and quantification may be carried out on mixtures of 100 components or more using amounts of less than one milligram by combined gas chromatography-mass spectrometry (g.c.-m.s.). In this technique a small quantity of the material to be analysed is separated and partially characterised by gas chromatography (g.c.) and structural information is obtained by mass spectrometry (m.s.).

Parallel advances have occurred in the other physical techniques: infrared spectroscopy (ir), ultraviolet-visible spectroscopy (uv-vis.) and nuclear magnetic resonance (n.m.r.) spectroscopy. Together with g.c.-m.s. these form a powerful array of analytical techniques which have solved many problems of long standing and stimulated entire new areas of investigation. Table 1.1. summarizes the sensitivities for several classical and modern physical methods. These figures present a rough index for comparison. The nature of the substances analysed and their purity also affect their detectability. In practice most measurements are carried out well above the limits of sensitivity wherever possible.

Before the advent of gas-liquid chromatography, fractional distillation was the most widely used gas phase analytical method. Extremely refined distillation techniques had been developed, especially in the petroleum industry, but these had several important limitations. The power of separating compounds of closely similar boiling points was inadequate; the methods usually required large samples (inappropriate for even the milligram scale); minor components of mixtures were not readily detected; and thermal decomposition was a frequent problem.

The efficiency of liquid chromatographic techniques for the analysis of complex mixtures of organic natural products became apparent from the extensive applications, in 1940-1955, of adsorption chromatography, especially in the steriod field. Characterisation of the separated components was generally based on classical methods together with absorption spectrometry. The concurrent development of liquid-liquid partition chromatography and of the allied technique of paper chromatography suitable for very small samples, led to the recognition of the value of chromatographic properties per se for characterising organic compounds. Chromatographic mobilities were employed as structurally indicative parameters.

A gap in techniques remained between the uses of liquid chromatography (primarily for samples of fairly low volatility) and of gas-solid chromatography (primarily for gaseous materials). This was filled by gas-liquid chromatography, introduced by A.J.P. Martin (Nobel Laureate) in collaboration with A.T. James in 1952. Although g.c. is capable of preparative use, it has been far more extensively applied purely to provide information about the composition of samples. The sensitivity of detectors permits the use of correspondingly small

Table 1.1. (continued)

Technique	Detection Limit	Identification Limit	Comments	Reference
Raman spectroscopy	10^{-1}-10^{-2}	—	Vibrational transitions: complements ir for structure and bonding, particularly useful for polymers.	10
Circular dichroism (c.d.) and optical rotatory dispersion (o.r.d.)	10^{-2}	—	Stereochemistry: conformational and configurational problems	11
Mass spectrometry				
Batch inlet (EI)	10^{-7}-10^{-6}	10^{-5}-10^{-4}	MW (empirical formulae by high resolution), structures, funcionality.	2
Direct insertion probe (EI)	10^{-11}-10^{-10}	10^{-9}-10^{-8}		
g.c.-m.s. (EI)	10^{-12}-10^{-11}	10^{-10}-10^{-9}		
Chemical ionization source (CI) g.c.-m.s.	10^{-12}-10^{-11}	10^{-10}-10^{-9}	MW, simplified spectra.	12,13
Field ionization source (FI) g.c.-m.s.	10^{-9}-10^{-8}	—	MW, simplified spectra.	14
Field desorption source (FD)	10^{-9}-10^{-8}	—	MW, useful for thermal sensitive and involatile materials.	15

samples, facilitating analysis of compounds of relatively high molecular weight and low vapour pressure. Where the general structural features of samples are already known g.c. can provide highly definitive information through correlations established with reference compounds, using a range of stationary phases and/or derivatives. Except for very simple compounds, g.c. data cannot yield reliable evidence of structure in the absence of prior information.

Table 1.2. offers a comparison of the efficiencies of principal methods of chromatography employed in organic analysis.[16,17] However, efficiency is not the sole criterion for usefulness. Thin layer chromatography has a long history of useful application, much recommended for its simplicity and versatility.[18] Liquid-liquid and liquid-solid chromatography have experienced a recent rapid expansion. Developments in pumping systems and other hardware have facilitated high pressure liquid chromatography (h.p.l.c.) which holds a hope of a uniquely flexible and powerful separation method.[19]

In mass spectrometry, direct measurement is made of the masses of ions arising from a molecule. The molecular ion, formed by loss of one electron, allows the molecular weight be inferred. Further structural evidence can be obtained from fragment ions - many of which result from interpretable modes of breakdown - and from characteristic isotopic ratios. At high resolution, the accuracy of mass measurement allows determination of the elemental composition of ions, thus greatly strengthening the assignment of structures. The diagnostic power of the direct dynamic coupling of the two instruments lies in the consistency which must obtain between the chromatographic retention time and mass spectrometric data for each component examined. The inherent advantages of g.c.-m.s. can be more expeditiously exploited by the use of computers for the continuous acquisition and evaluation of the data.

For a particular sample a variety of techniques may be usefully deployed in the analysis. For example, studies of a range of derivatives by t.l.c. and g.c. can yield provisional evidence of probable structural types. For biological samples of complex composition, fractionation by chromatographic and classical procedures into groups of related functional type is almost invariably necessary: each group can then be converted to the most suitable derivative, and analysed under the most appropriate g.c.-m.s. conditions.

Table 1.2. Chromatographic efficiencies of common methods.[16,17]

Technique	Column type	Mean particle size	N_{eff}/t
Thin layer chromatography	—	2-20	0.05
Liquid-liquid chromatography	Classical packed	150	0.05
	Silica gel	20	2
	Porasil	31	2
	Zipak	27	8
	"Infinite diameter, CSP support"	27	13
	Silica microspheres	5	23
Gas-liquid chromatography	Classical packed	130	10
	Porous-layer beads	90	50
	Porous-layer open tubular	—	
	Wall-coated open tubular	—	100

N_{eff}/t is an expression reflecting the relative performances of the different systems (number of effective theoretical plates per second).

$$N_{eff} = n.k'/(1 + k')^2$$

N, the number of theoretical plates, and k', the partition ratio are defined in Sections 1.2.2. and 2.2.3.

1.2. Gas Chromatography

1.2.1. Introduction. Gas chromatography is a technique for the separation and characterisation of compounds according to their distribution between a mobile gas phase ("carrier gas") and a stationary phase. If the latter is a solid, the distribution usually depends on adsorption effects. In gas-liquid chromatography (hereafter simply g.c.), the stationary phase is a liquid or gum, coated in a thin film either on the inner wall of the chromatography column (open-tubular g.c.), or on a finely-divided inert granular support (packed-column g.c.). In general, conditions are designed to minimise adsorption, and to achieve partition chromatography, in which the mobility of a compound is determined by its relative solubility in the mobile and stationary phases. Since the sample concentration in the gas phase is primarily dependent upon its vapour pressure at the operating temperature, the gas-chromatographic behaviour of a compound is broadly related firstly to its molecular weight and secondly to its interaction with the stationary phase. Gas-liquid chromatography is thus complementary to the techniques of liquid-liquid, liquid-solid, and liquid-gel chromatography in which separations are generally less sensitive to molecular weight.

The introduction of g.c. in 1952[20] led to a large area of applications, in which g.c. has transcended the scope of earlier techniques. Its advantages can be briefly summarised as follows:

(a) Efficiency and versatility of separation. The resolving power of g.c. exceeds that of all other chromatographic techniques (see Table 1.2.), with the partial exception of high-pressure liquid-solid chromatography. Many separations not demanding a high degree of resolution can be achieved with simple columns and very short analysis times.

(b) Sensitivity of detection. Most gas chromatographs are equipped with hydrogen flame ionisation detectors, allowing the convenient study of samples of 0.1 - 1 μg (10^{-7}-10^{-6} g). Analysis at the 1 pg (10^{-12} g) level is possible with selective detectors and particular compounds.

(c) Power of characterisation. Compounds are characterised by their retention volumes under given conditions: these are highly reproducible, and for very simple compounds the retention volumes on several stationary phases may afford definitive identification. In general, characterisation is based on careful comparative studies of

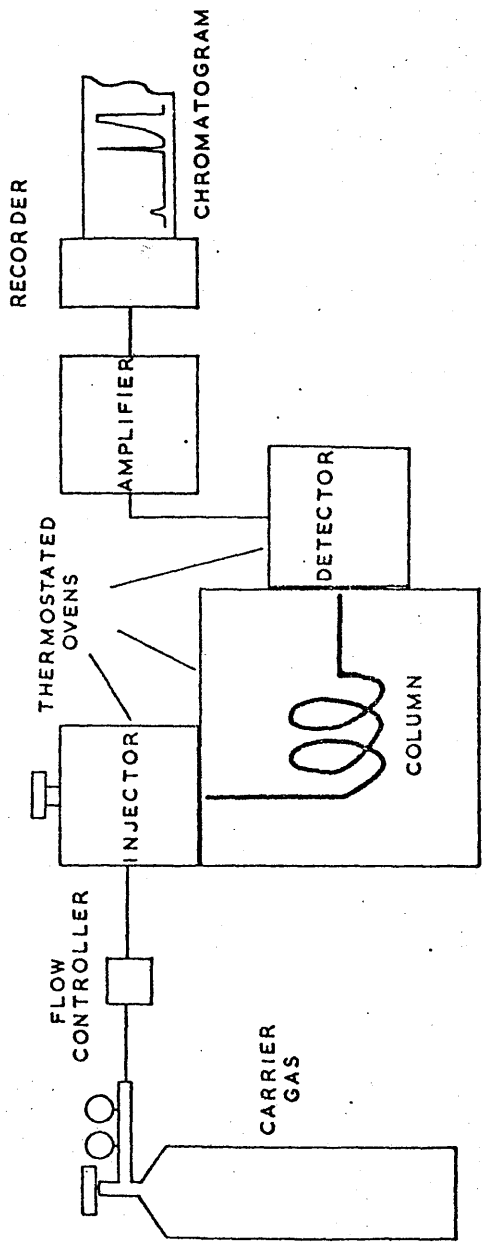

Figure 1.1. Schematic diagram of the components of a conventional gas chromatograph.

various derivatives and on correlations with reference compounds.

(d) Quantitative applications. The validity of each estimation can be monitored by the simultaneous qualitative analysis of the sample components: this affords a major advantage over many spectrophotometric or spectrofluorimetric analyses and over radio-immunoassay techniques.

(e) Range of application. The high sensitivity of detection methods, mentioned above, is such that only about 1μg of sample need be volatilised. Accordingly, g.c. is applicable to compounds of high molecular weight (1000-2000) provided that functional groups are modified (where necessary) to improve volatility or stability. Even short-lived compounds can often be analysed satisfactorily, by virtue of the inertness of the carrier gas, the short time of the analysis, and the possibility of dispensing with extensive preliminary purification of samples.

The chief limitations of g.c. are its inapplicability to involatile samples (except via pyrolysis) and the fact that it yields no direct information on the molecular weight or structure of a substrate.

1.2.2. Basic principles. The gas chromatograph is an analytical instrument which provides a simple, rapid, sensitive and accurate method for the separation, identification and determination of volatile compounds. Its construction is shown in Figure 1.1. An inert carrier gas of high purity is passed, via a flow controller, to the injector. Sample, as solid, liquid or solution, is vaporised in the heated injector and swept as a narrow concentrated band on to the column without serious disruption of carrier gas flow. The separation process occurs under controlled temperature conditions, most often isothermal or in a linear temperature programme, and the column effluent is passed on to the thermostated detector. The signal from the detector is converted to an analogue voltage by an amplifier or other electronic device, and this is recorded as a function of time by a strip chart potentiometric recorder. The area of the peak, the integral of the analogue signal, is proportional to the quantity of the component.

The separation of two compounds in g.c. depends on the difference in their distribution coefficients with respect to the mobile and stationary phase at a given temperature. In practical terms this requires the selection of the most suitable stationary phase.

Separating power, usually termed resolution, is further determined by the column efficiency: this is reflected by the degree of broadening of the initially compact band of vapour as it passes through the column.

The rate of peak broadening is a function of column design and operating conditions, and is quantitatively related to the "height equivalent to a theoretical plate" (HETP),[21] a concept derived from distillation theory. The number of theoretical plates (N) may be conveniently calculated by the expression

$$N = 5.54 \, (w_{1/2}/t_{dr})^2 \qquad \text{(eq. 1.1.)}$$

where $w_{1/2}$ is the peak width at one half height and t_{dr} is the uncorrected retention time. The HETP is then obtained by:

$$HETP = L/N \qquad \text{(eq. 1.2.)}$$

where L is the length of the column, normally in cm. This is the preferred measure of column efficiency, and allows comparison of different column lengths under specified conditions of temperature and gas flow rate, sample type and amount, and stationary phase.

In gas-liquid chromatography on packed columns, several theories have been developed to explain effects leading to peak broadening. The rate of theory of van Deemter et al. is perhaps the most useful. The theory relates column efficiency measured as average HETP to the linear gas velocity ($\bar{u}$) as:

$$HETP = A + B/\bar{u} + C\bar{u} \qquad \text{(eq. 1.3.)}$$

The terms on the right hand side of this equation represent the three principal causes of peak broadening.[22]

The multiple path term (A) arises from the tortuous narrow passages in the bed of the particles which constitute the packed column. These paths are devious and of different lengths, and result in an approximately Gaussian spreading of the peak as it traverses the column. The molecular diffusion term (B) results from the diffusion of solute molecules longitudinally. The resistance to mass transfer term (C) describes the ease of passage of solute molecules between the stationary liquid and moving gas phases.

Since the carrier gas is compressible, the linear gas velocity is not constant throughout the column. Thus the average height

equivalent to a theoretical plate ($\overline{\overline{HETP}}$) is determined for an average linear gas velocity ($\bar{u}$). The contribution of molecular diffusion (B) increases with decreasing flow rate and with decreasing density of carrier gas. Resistance to mass transfer (C) increases with increasing flow rate and density of carrier gas, and with increasing viscosity and thickness of the film of stationary phase. Therefore, an optimum flow rate is a compromise between the B and C terms. The choice of carrier gases of various densities is nearly self-cancelling and is normally dictated by the requirements of the detector. The A term is constant for a given column and is independent of flow rate. In packed column g.c., optimum performance is obtained with small uniform inert support particles, coated with a uniformly thin film of phase and evenly packed in columns of small diameter.

In open-tubular columns the multi-path term (A) disappears, offering a potential advantage of these over packed columns. In practice, however, the plate heights are of the same order for columns of either type.[23] The comparatively unrestricted gas flow in open-tubular columns permits the use of a wide range of column lengths. High numbers of theoretical plates can be obtained by the use of long columns; alternatively, shorter columns are effective for rapid analyses. Thus it is the "open" nature of these columns compared with packed columns that is most significant, rather than the generally narrow diameter which the popular term "capillary column" might imply. Columns in which the phase forms a film on the wall of the column are briefly designated as wall coated open-tubular (WCOT). The surface area available for stationary phase may be increased by modifying the inner wall of the column to produce a porous layer open-tubular (PLOT)column or by depositing a thin layer of support material to produce a support coated open-tubular (SCOT) column: these modifications provide increased sample capacity.

1.2.3. Features of the technique.

(a) Sampling. Gas samples may be injected via a gas-tight syringe or by means of a mechanical inlet manifold. Liquids or solids in solution may be conveniently injected by means of a micro-syringe through a self-sealing septum port. With injections in a volatile solvent, the early part of the chromatogram is obscured by the "solvent peak". This occupies part of the chromatogram: moreover the solvent may also have deleterious effects on the column or the

detector. For samples that are not too volatile, these problems can be avoided by the use of a dry sampling technique.

(b) Thermostated areas. The operating temperature of the gas chromatographic column depends on the dimensions of the column, the particular stationary phase and its concentration. These are selected to suit the samples being analysed. In the case of the injection device the temperature must be sufficient to allow rapid vaporisation of solid or liquid samples but not so high as to cause decomposition. The column temperature must be controlled within very narrow limits. Isothermal and temperature programmed modes may be selected according to the requirements of the analysis. The temperature of the detector is determined by its normal operating limitations and by the temperatures of the rest of the system. Close attention to the control of the temperature of all parts of the system is necessary, especially when easily condensible samples are being studied.

(c) Stationary phases. The column is the heart of the chromatographic system. In addition to the various column types mentioned above, great flexibility in analysis is obtained by the choice of the appropriate stationary phase from among more than 300 that are available. Where column efficiency is insufficient to obtain a satisfactory separation, phase selectivity may often succeed. As a general rule, stationary phases should not be widely different in polarity from the samples being chromatographed. Where compounds of different classes but similar boiling points are to be separated, a phase of appropriate selectivity must be used. The chromatography of a given sample on several distinctive phases is often of great importance in characterisation and in confirming homogeneity.

(d) Detectors. Most analytical work is carried out on samples ranging from 1ng to a few µg. Thus, sensitive nonspecific detectors are required that are linear in response and insensitive to changes in flow rate and temperature. While differing in operating principle, the argon ionisation detector and the flame ionisation detector have been found to be very suitable in these respects. In the argon detector the sample remains practically unchanged, while in the flame ionisation detector it is largely destroyed by combustion in a hydrogen flame. The non-destructive thermal conductivity detector is applicable wherever the highest sensitivity is not needed.

There is also a useful place for detectors that are designed to

respond to specific classes of compounds. The electron capture detector, which is responsive to halogenated compounds and conjugated carbonyl compounds, and alkali flame ionisation detectors, which are largely specific for phosphorus and nitrogen compounds, are good examples of such devices. Radio-activity detectors are useful for studies involving isotopically labelled materials. Multi-element detectors or several specific detectors in parallel may be valuable for special analytical applications.

(e) Quantitative analysis. In general, the recorded peak areas are a function of the amounts of sample eluted. In isothermal gas chromatography, conveniently measured relative peak heights are as satisfactory as relative peak areas for the evaluation of relative concentrations. However, in programmed temperature gas chromatography it is advisable to determine peak area. These areas may be evaluated in a number of ways: manual methods such as triangulation or excision and weighing are effective, but mechanical or electronic integrators are much faster and are essential for routine quantitative analysis, especially of complex mixtures.

Analytical calibration may be undertaken directly or by using a known concentration of a reference compound (of different but comparable retention time) as an internal standard. The latter procedure improves precision in analyses based on the injection of solutions by micro syringe, by compensating for changes in the volumes injected. The practically instantaneous vaporisation that occurs in a properly designed injection system preserves the original relative concentration of solutes.

Detectors must be checked for their linear response over the concentration ranges in question. Calibration should be carried out for each particular compound since there may be marked variation in "response factors". The flame ionisation detector is particularly suitable as a general detector by virtue of its linear response over a very wide concentration range.

1.2.4. Standardisation of retention behaviour. Largely because of the long-term stability of gas chromatographic columns (column temperature and flow rate being controllable within narrow limits) reproducible conditions of separation are easily attainable in gas chromatography. While separation is the outstanding feature of the technique, retention data provide powerful ancillary information. In isothermal chromatography, retention data may be recorded as dimensionless

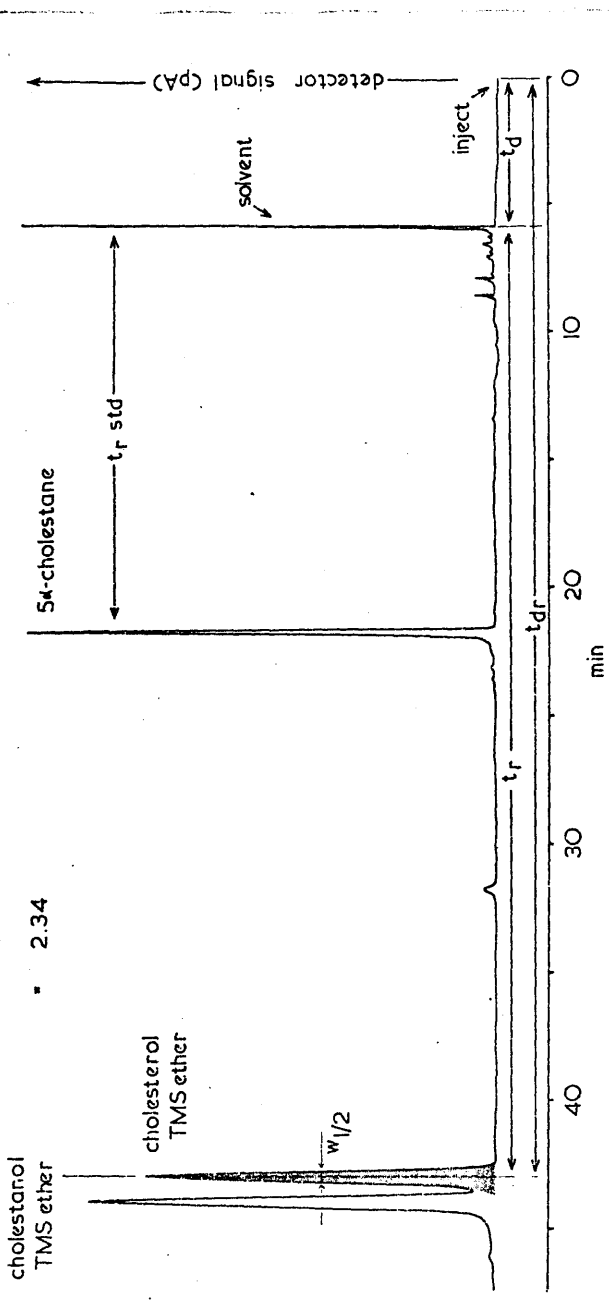

Figure 1.2. Chromatogram of the separation of cholesterol and cholestanol as trimethylsilyl (TMS) ether derivatives. 5α-Cholestane was included as an internal standard and the relative retention time (RRT) was computed for 5α-cholestan-3β-ol TMS ether. The trace is labelled illustrating the typical features of a gas chromatogram. Chromatographic conditions: 50m x 0.5mm i.d. Silanox-type (PLOT) glass OV-1 column installed in a Pye Model 104 gas chromatograph equipped with flame ionisation detector, temperature 265°C, helium carrier gas 6ml/min.

numbers derived from relative retentions with respect to a conveniently selected internal standard. These ratios, which are sensibly constant for each compound (for a given stationary phase at a particular temperature) are characteristic physical properties, and may be applied effectively as indicators of structural features.

Figure 1.2. depicts a chromatogram of the separation of cholesterol (5-cholesten-3β-ol) and 5α-cholestan-3β-ol as their trimethylsilyl (TMS) ether derivatives. 5α-Cholestane is included as internal standard and the main features are indicated. The sample is characterised by its retention volume corrected for the "dead" volume of the column. For constant gas flow rate it is not necessary to compute volumes, retention time (t_r) is obtained by subtracting dead time (t_d) from the uncorrected retention time (t_{dr}). The dead time (t_d), the time required for an unretained component to traverse the column, is here approximated by the time required for hexane (solvent) to emerge. The calculation of relative retention time (RRT), a numerical expression of retention behaviour compared to 5α-cholestane, is illustrated in Figure 1.2.

(a) Behaviour of homologous series. At constant temperature it has been shown that for compounds forming a homologous series, log (retention volume) is a linear function of a number of carbon atoms. This relationship is very precise for all but the few early members of simple carbon-chain series. Useful correlations hold also for quasi-homologous compounds such as steroids. The predictive value of linear plots is very good, and graphs of log (retention time) against carbon number have been extensively applied in structural analysis. Figure 1.3. shows the behaviour of homologous n-alkanes, n-alkanols and n-alkanol TMS ethers.

The slopes of the plots are approximately equal over a relatively wide span, and the introduction of a particular functional group in a particular location is associated with a characteristic logarithmic increase in retention. Similar retention increments may be observed in series of analogous compounds similar in structure in the region of the site of substitution. The actual magnitude and direction of such changes is, of course, dependent on the interaction of substituent groups with the stationary phase. Qualitative assessment of functional groups is greatly facilitated by comparisons of retention behaviour on columns of differing polarity. In linear temperature programmed gas chromatography at moderate rates of temperature rise (2-10°/min) it is found that consecutive members of a homologous series are eluted at approximately equal intervals.

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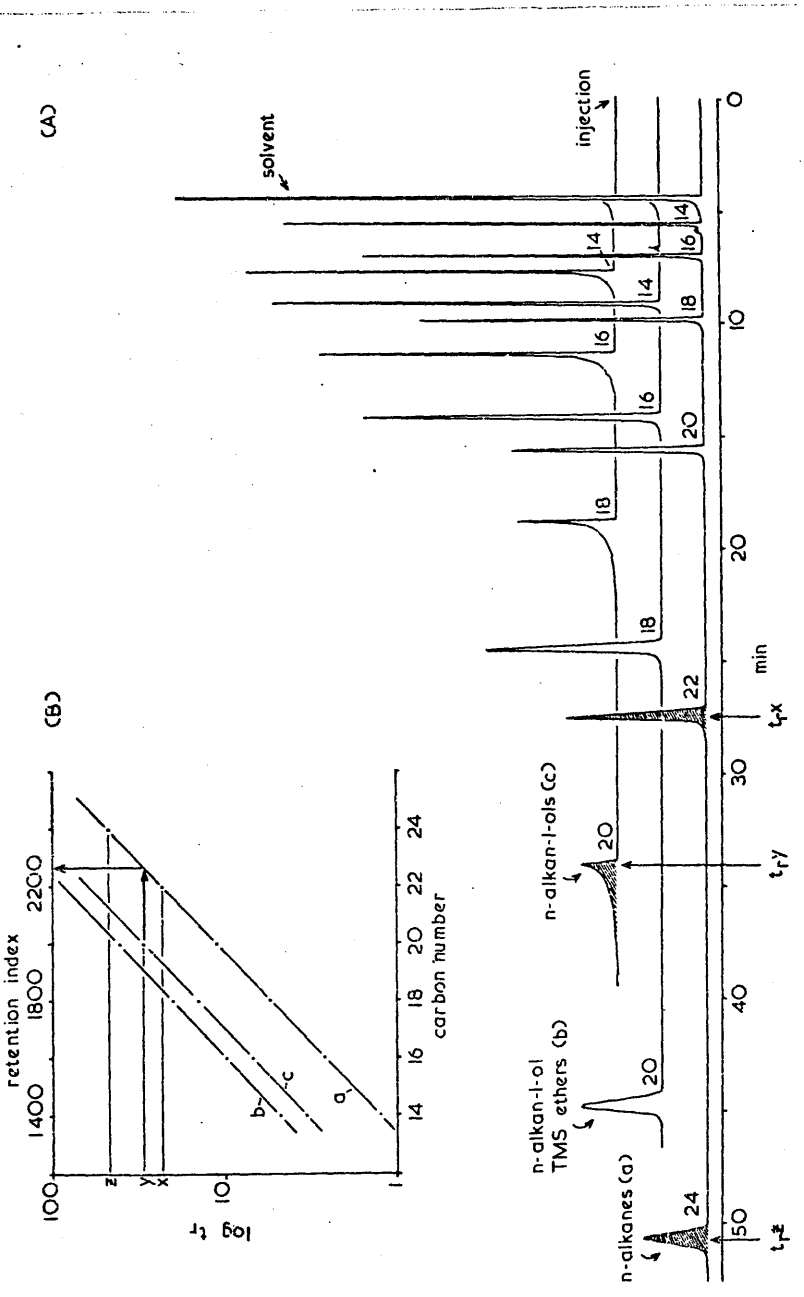


Figure 1.3. (A) Composite of three chromatograms of mixture of normal alkanes of even carbon number from C_{14} to C_{24} (a), the normal alkan-1-ols of even carbon number from C_{14} to C_{20} as the free compounds (c) and the trimethylsilyl (TMS) ether derivatives (b). Chromatographic conditions: 50m x 0.5mm i.d. Silanox-type (PLOT) glass OV-1 column installed in a Pye Series 104 gas chromatograph equipped with a flame ionisation detector, temperature 200°C, helium carrier gas 10ml/min.
(B) Semilogarithmic graph demonstrating the linear relationship between the log of retention time (t_r) and carbon number in homologous series of n-alkanes (a), n-alkan-1-ols (c) and n-alkan-1-ol TMS ether derivatives (b). Bold arrows demonstrate the Kovats retention index of n-eicosan-1-ol (y) as 2270, determined by the bracketing even numbered n-alkanes, docosane (x) and tetracosane (z).

The retention time is thus a nearly linear function of carbon number under these conditions.

(b) Group retention factors. Clayton examined the retention of a large number of steroids and defined group retention factors as a ratios of retention times of mono-substituted and corresponding unsubstituted steroids.[24] He showed that steroids differing in structure remote from the site of substitution showed similar factors. The observed relative retentions are approximated by the product of group retention factors (for each functional group) and the relative retention of the parent hydrocarbon, i.e.,

$$RRT = RRT_{nucleus} \times k_a \times k_b \times \ldots\ldots \qquad \text{(eq. 1.4.)}$$

where RRT is the retention of the substituted steroid. $RRT_{nucleus}$ that of the parent hydrocarbon and k_a, k_b, etc. are group retention factors. For steroids containing more than one substituent the correlation is most satisfactory where the substituents are far apart. The regularity of such correlations, and their value in detailed structure elucidation, has been substantiated by many applications. The logarithms of group retention factors may be used as additive parameters.

(c) Retention indices. Relative retention values expressed with respect to a single reference compound are markedly dependent on temperature. This is due to the variation of partition coefficients of different types of compound with temperature. The retention index system devised by Kováts, which employs the members of the n-alkane series as fixed reference points, is less temperature dependent.[25] In isothermal chromatography the linear plot of $\log (t_r)$ for homologous alkanes against carbon number defines a scale such that the retention index of n-alkane, $n\text{-}C_nH_{2n+2}$ is 100n. Retention indices are then obtained by simple interpolation. This may be expressed algebraically for compound x of retention time $t_r(x)$ as:

$$I_{x\ \text{temperature}}^{\%,\ \text{stationary phase}} = 100x\frac{\log t_r(x)-\log t_r(z)}{\log t_r(z+1)-\log t_r(z)} + 100z \qquad \text{(eq. 1.5.)}$$

where t_r is the adjusted retention time, z and (z+1) are the carbon numbers of the bracketing n-alkanes i.e., $t_r(z) < t_r(x) < t_r(z+1)$. Retention indices are easily obtained graphically as illustrated in Figure 1.3. In linear temperature programmed gas chromatography a similar interpolation may be made employing the linear plot of t_r against carbon number (or carbon number x 100).

The advantage of the retention index system is its consolidation of retention data in a standard numerical form, relatively invariable over a moderate range of temperature (typically 1 index unit per ^{o}C). Qualitative assessment of structure and functional groups may be speedily accomplished by comparison of absolute values of retention indices and of their increments. By rough estimate the assignment of an unknown component to one of the 600 or so retention index 'windows' is possible in a normal chromatogram. This does not serve to distinguish the many compounds which might coincidentally elute within the observed interval. Complete analyses must employ other stationary phases, other ancillary techniques and confirmation by other methods is usually necessary. Among the most powerful ancillary techniques of gas chromatography is the dynamic combination with mass spectrometry.

1.3. Mass Spectrometry

1.3.1. Basic principles. Mass spectrometer design and operation has in recent years undergone much elaboration and modification. Extensive reviews of operating principles and techniques of interest to the organic chemist has been presented by Roboz,[26] Watson,[27] and McFadden[2].

In brief, the technique permits direct measurement of the masses (or strictly the ratio of mass to charge: m/e values) of ions produced by electron impact or other means from the sample vapour. Frequently one of these ions results simply from the loss of one electron, and thus indicates the molecular weight. The m/e values and abundance of other ions provide information on probable structural components, and the total "mass spectrum" is a characteristic of the compound, though isomeric compounds often give very similar mass spectra. With instruments of high resolving power, masses can be determined precisely enough to permit assignments of elemental formulae to be made. Thus mass spectrometry is an excellent technique for obtaining evidence of molecular structure from samples of the order 0.1 - 1.0μg. The separation of components of mixtures can, however, be effected only crudely, in general, by fractional evaporation into the ion source region.

Most mass spectrometers depend on the same operating principle: sample molecules are vaporised into a vacuum and ionised. The energised molecular ions may decompose to fragment ions which are

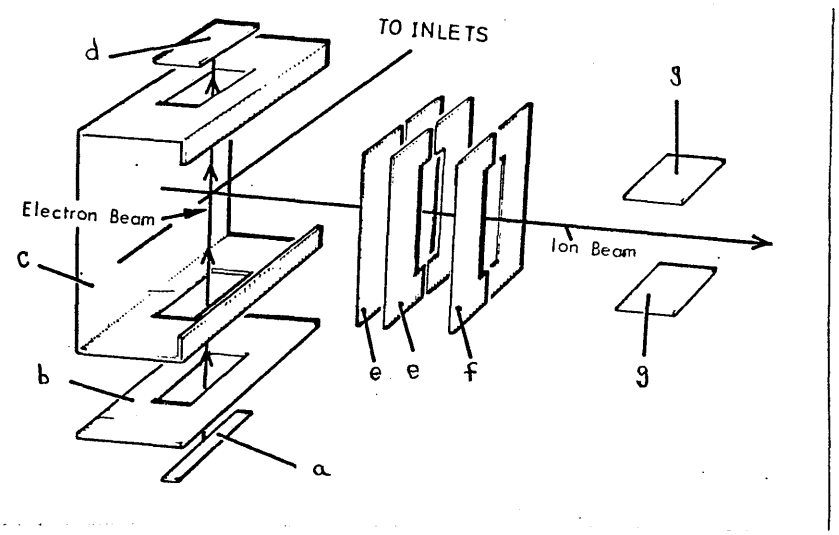


Figure 1.4. Schematic of ion optics of electron-impact ionisation source, voltages for 20eV ionising energy: (a)rhenium filament 3480V, (b) shield 3480-3490V, (c) ion chamber or box 3500V, (d) trap 3650V, (e) extraction plate 3490V, (f) focusing lens 2500V, (g) deflection plates 500V. Diagram reproduced by permission of LKB Instruments Ltd., South Croydon.

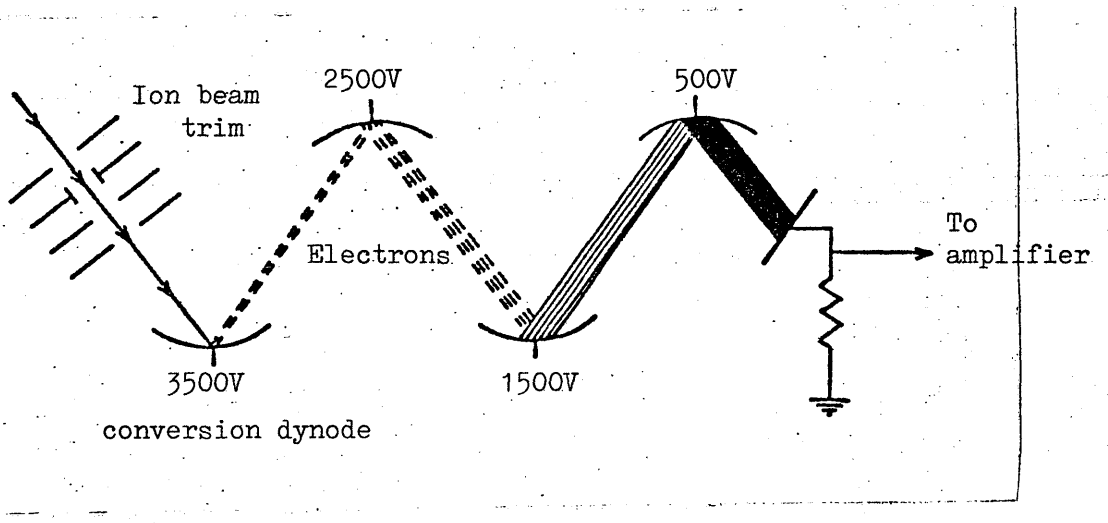


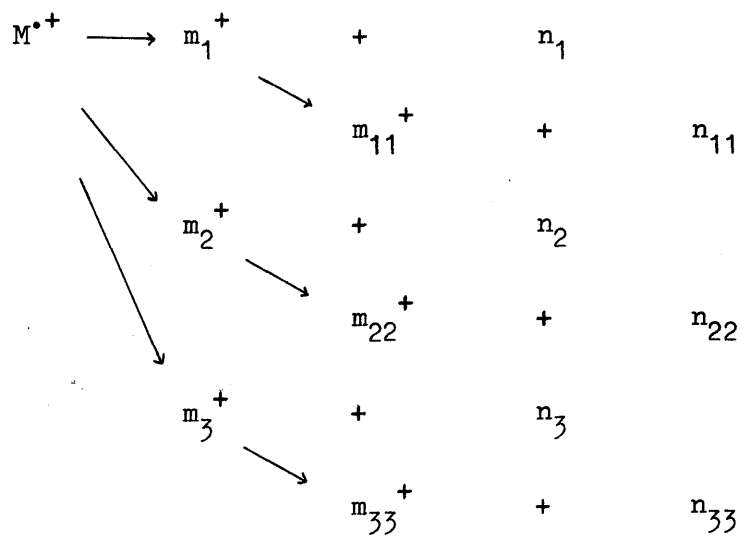
Figure 1.5. Schematic diagram of a three stage electron multiplier.

separated according to mass. The relative abundances of the various ions are measured and this information is used to identify the sample. The conventional electron impact source employs a narrow magnetically collimated electron beam from a heated rhenium wire filament as the ionising medium. The energy of the bombarding electrons can be adjusted by varying the potential between the filament and the trapping anode, generally from 5-100 volts, thereby providing control over the ionisation and fragmentation processes. Figure 1.4. represents the ion optics of an electron-impact source.

Most organic molecules require 6-12 eV (electron volts) energy for producing molecular ions by loss of electrons.

$$M \longrightarrow M^{\bullet +}$$

An increase of 5-10 eV over the ionization potential promotes fragmentation by the rupture of the chemical bonds. The molecular ions may decompose to give primary fragment ions (m_1^+, etc.) which may, in turn, break down to produce secondary fragment ions (m_{11}^+, etc.) and so on until sufficient energy has been carried away by the neutral fragments (n_1, etc.) to leave stable fragments:



Most of the ions produced are singly-charged but a few may be multiply-charged by further electron loss. The population and distribution of positive ions reach a plateau well below the 70 eV normally employed. Sensitivity of the instrument may be conceded for the advantage of less fragmentation and more prominent molecular and primary ions at lower electron energy i.e. 20 eV. After ionization the resulting assemblage of positively charged molecular and fragment ions is focused into a well-defined ion beam and

accelerated out of the source by a series of plates that are negatively charged with respect to the ionizing chamber. All the ions are accelerated across the same potential, eV (e = electronic charge, V = accelerating voltage) and acquire the same kinetic energy, $\frac{1}{2}mv^2$ (m = ion mass, v = ion velocity). However, since the ions have different masses, they have different velocities upon entering the magnetic field. The ion paths have different radii of curvature and the total beam is dispersed into a spectrum according to the ratio of the mass to charge ($\underline{m}/\underline{e}$).

At a given field strength ions of a specific mass are brought to focus and the potential and kinetic energies are equal :

$$eV = \tfrac{1}{2}mv^2, \qquad \text{(eq. 1.6.)}$$

and the centrifugal and centripetal forces are balanced in the region of the magnetic field:

$$HeV = mv^2/R, \qquad \text{(eq. 1.7)}$$

where H is the magnetic field strength (0-13 kilogauss) and R is the radius of curvature of the ion trajectory. So it can be seen

$$m/e = R^2H^2/2V \qquad \text{(eq. 1.8)}$$

The entire mass spectrum may be scanned across the detector slits by continuously varying the magnetic strength or the accelerating voltage. Singly-charged ions are assigned mass-to-charge ($\underline{m}/\underline{e}$) values corresponding to the sums of the atomic weights (carbon = 12.0000) of the constituent atoms. Multiply-charged ions are observed at $\underline{m}/\underline{e}$ values corresponding to the appropriate fraction of their masses.

Positive ions arriving at the collector slit are detected and amplified by an electron multiplier. Figure 1.5. shows a schematic of this device. The principle of secondary electron emission is used to effect amplification. The positive ion beam is directed to the first plate, the conversion dynode, where it is converted to an equivalent electron current by a secondary electron emission. These secondary electrons are accelerated and focused onto a second dynode and amplification is achieved by a "cascade effect" from dynode to dynode. The number of electrons ejected from a dynode is always greater than the number impinging on it. A multi-stage device, each stage connected to a

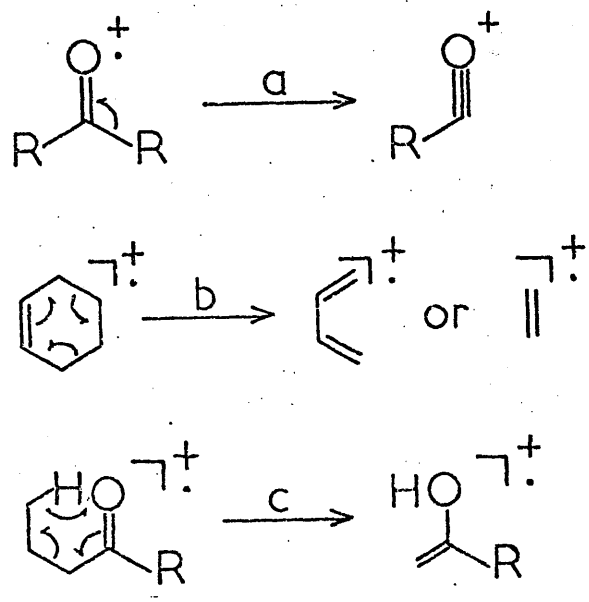
successively higher potential may produce a gain of 10^7 for a total voltage of 3.7kV. The final anode at ground potential is connected to a conventional amplifier. The output of this multiplier preamplifier is transferred via an impedance matching galvonometer amplifier to an oscillographic recorder. A semi-permanent oscillogram on photosensitive paper is produced by light reflected from three mirror galvonometers having relative sensitivities 1:10:100. Mass calibration of this analogue record of ion intensities is obtained by a series of grid marks produced simultaneously on a fourth galvonometer trace. This "mass marker" which functions only for magnetic scanning is obtained from a Hall cell[28] in the magnet gap which produces a potential proportional to the field strength. This potential is converted to a digital output and calibrated in mass units against perfluorokerosene as mass standard. This independent calibration device is sensitive to temperature and positional changes and may require frequent recalibration.

The ion beam may alternatively be mass analysed on non-magnetic principles by a radio-frequency quadrupole electric field. The information thus obtained is generally reduced to digital form by measuring peak height, representing "abundance" of each m/e value and recording its percentage of the most abundant ion in the spectrum. These data are then reconstructed into a graph of the original spectrum, showing both relative intensity, and per-cent total ionisation, for each m/e value.

1.3.2. Structural information. Occasionally, ions of mass m_1 decompose in the field-free regions between the source and the multiplier collector. This process, which is most likely to occur as the ion beam passes the focus plates or final accelerating plates, produces fragment ions of mass m_2 together with neutral fragments. The neutral fragments carry away a fraction of the kinetic energy, so that ions formed in the field-free region possess less kinetic energy than ions of the same mass produced in the ion source. These ions are brought to focus at an apparent mass, m^*, which is approximately related to m_1 and m_2 by:

$$m^* = m_2^2/m_1 \qquad \text{(eq. 1.9.)}$$

The neutral particles may carry away varying amounts of energy from the ions m_1. Therefore, the m/e values of the ions of apparent mass m^* are dispersed and appear in the spectrum as diffuse broad peaks.



Scheme 1.1. Typical electron impact induced fragmentation; simple cleavage of ketones (a), retro-Diels-Alder fragmentation (b), and the McLafferty rearrangement (c).

These "metastable" ions are frequently useful, especially in the absence of high-resolution data, in showing fragmentation transitions in the spectrum.

The sort of information obtainable from such a mass spectrum depends largely on the type of sample and the conditions under which the spectrum was obtained. Most materials give a molecular ion which is often enhanced in relative intensity with a moderate ionising energy (12-15eV). Electron impact-induced fragmentation is seldom a random process. All but the most simple of molecules contain bonds of substantially differing lability. Preferred modes of fragmentation may be simple bond fission, at in the α-cleavage of ketones (Scheme 1.1a.). More complex modes may involve more extensive electron rearrangement as in the "retro-Diels-Alder" fragmentation of cyclohexene-type systems (Scheme 1.1b.) or atomic rearrangement, as in the "McLafferty rearrangement" (Scheme 1.1c.). Together with these more predictable and explicable fragmentations, there occur a large number of less specific cleavages. The complete spectrum under specified conditions forms a characteristic "fingerprint" of the molecule.

Since the inception of the technique, spectra of many thousands of organic compounds have been recorded, compiled and published in numerous reference works[29,30] and collections.[31-37] A useful guide to these has recently appeared.[38] Empirical analysis and digestion of this data has produced many insights into the correlation of mass spectra with the structure of the compounds.[39-43] A comprehensive volume on the use of mass spectral methods in biological investigations includes extensive reviews covering fatty acids, lipids, steroids, bile acids, carbohydrates, terpenes, amino-acids, oligopeptides, nucleic acids, antibiotics, vitamins, hormones, tetrapyrroles, alkaloids, flavour components and semiochemicals.[44] Gross structural features are easily distinguishable and positional isomers provide mass spectra which differ substantially. In advantageous cases stereoisomers and epimeric pairs may be distinguished. However, for all but the simplest of examples deduction of the structure from only mass spectral data is impracticable. Comparison of spectra with standards and reference spectra of related materials must be obtained as supportive evidence. Data from other physical techniques are also generally desirable.

1.3.3. Special techniques. In addition to the commonly used

method of ionisation by electron bombardment, a number of alternative ionisation techniques are available. Energy in excess of that required for ionisation leads to various fragmentations some of which are specific to the overall structure. However, when fragmentation predominates, as in many large, complex or sensitive molecules, the important information of molecular weight, in the form of molecular ions, may be unclear or lacking. As already noted, electrons of lower energy may be used, but at the expense of sensitivity and not always with improvement of molecular ion abundance. "Soft" ionisation techniques impart little excess energy above ionisation energy and so minimise fragmentation of molecular species.

Chemical ionisation (CI)[12,13] mass spectra result from ion-molecule reactions between the sample vapour and ions produced by electron bombardment of a reactant gas (e.g. methane, isobutane,, ammonia or water) at about 1 torr. Virtually all ionisation due to electron bombardment occurs in the reactant gas which may itself undergo ion-molecule reactions to form secondary and higher-order ionic products, any of which may in turn ionise sample molecules. This results in fragment and product ions characteristic of the reactant gas and the sample. Ions containing essentially intact molecular species frequently account for a high percentage of the sample ionisation.

Field ionisation (FI)[45,46] and field desorption (FD)[47,48] mass spectrometry rely on the removal of an electron from a molecule by a high electric field through the action of the quantum-mechanical tunnelling effect. In the case of the latter technique the sample need not be in the vapour phase but can be on the surface of an ion emitter. The stability of large organic molecules of biological importance to electron impact or thermal energy is often so low that no ions from undecomposed species can be detected in conventional mass spectra. In such cases the molecular ions are usually detectable in the FI and FD spectra. Derivative formation for highly polar materials becomes a correspondingly less important consideration. These developments have been compared by Fales *et al.*,[49] in respect of the analysis of biologically important compounds.

High resolution mass spectrometry employing electrostatic energy focusing and magnetic dispersion allows accurate mass measurement of ions by "matching" reference and unknown ions. With

sample and reference compounds simultaneously in the ion source, the accelerating voltage at constant magnetic field is altered sufficiently to bring reference and unknown ions alternately to focus on the detector. A reference ion of precisely known mass (m_1) from the reference compound is brought to focus at normal accelerating voltage (V_1). The accelerating voltage is reduced by a precisely known amount (to V_2) by a precision decade potentiometer to bring a higher mass unknown ion (m_2) into focus. The exact super-position is established on a long-persistence oscilloscope screen while the beam is modulated to "sweep" the two ions. Under these conditions the relation

$$\frac{m_2}{m_1} = \frac{V_1}{V_2} \qquad \text{(eq. 1.10)}$$

is true and the ratio, read from the decade, multiplied by the reference mass affords the unknown mass. Although this technique is primarily designed for instruments of high resolving power, affording mass measurements accurate to 0.01amu, it may also be useful with those of lower resolution employing only magnetic analysis. However, in the latter case the technique is effectively limited to true singlet ions of narrow energy profile (in practice almost exclusively molecular ions): in favourable circumstances, accuracy may be sufficient to distinguish between two elemental compositions.

"Tracer" methods of analysis measuring natural and synthetic isotopic abundance ratios may be followed by mass spectrometry for any element for which two stable or long-lived isotopes exist. Dilution analyses employing stable isotopes require high isotope enrichment of reference compound for the highest sensitivity. The use of mass spectrometry often simplifies the analysis. So long as the isotopic abundance ratios can be measured to the required degree of accuracy and there is no interference from other substances at the same masses, the sample preparation stages may be simplified or abandoned. In sorting wanted ions from unwanted ions the mass spectrometer itself effects a purification step.

Derivatives may be used to improve volatility and stability and to induce mass increments and informative fragmentations in the mass spectrometric analysis of compounds of high molecular weight.

1.4. Gas Chromatography-Mass Spectrometry

1.4.1. Basic considerations. An outline has so far been given of the virtues of gas chromatography and mass spectrometry as separate techniques. The direct combination of a gas chromatograph and a mass spectrometer constitutes an analytical system of unparalleled capability. Mass spectra obtained rapidly on components emerging from a gas chromatographic column afford informative correlations of mass spectrometric and chromatographic data. Full use is made of the separating power of g.c. together with the structural information derivable from m.s. This gives exceptional power of discrimination between closely similar structures. Two themes have recently been of particular significance in the application of the technique: firstly, the extremely high sensitivity that can be achieved in detection and quantitative estimation, by using the mass spectrometer as a mass selective detector; secondly, the application of derivatisation to control both the chromatographic and mass spectrometric behaviour of compounds with the aim of enhancing the informative elements in the data.

Apart from sample volatility, purity is the most important consideration for a sample to be analysed by mass spectrometry. The interpretation of mass spectra relies on the assumption that all the ions of the spectrum arise from a single molecular species. A few abundant fragment ions from a minor impurity can create confusion. Classical separation methods and preparative chromatographic techniques may often be difficult and time consuming. Gas chromatographic fractions may be trapped and analysed individually through the direct inlet of the mass spectrometer.[50,51] However, in dealing with a complex mixture the isolation of minor components may be impractical. The gas chromatograph and the mass spectrometer both require samples in the vapour phase. A dynamic combination would be advantageous in that components separated by the chromatograph may be presented sequentially to the mass spectrometer for identification. Chromatographic and mass spectral data are correlated. The subtle differentiation inherent in the chromatographic separation suits well the mass spectrometer requirement for sample purity. The mass spectrometer offers structural information on the amounts of sample material contained in a normal chromatographic injection. The difficulty and tedium of prior fractionation is obviated. Samples which were previously not suitable for analysis because of limited amounts of sample, complexity of the sample, or

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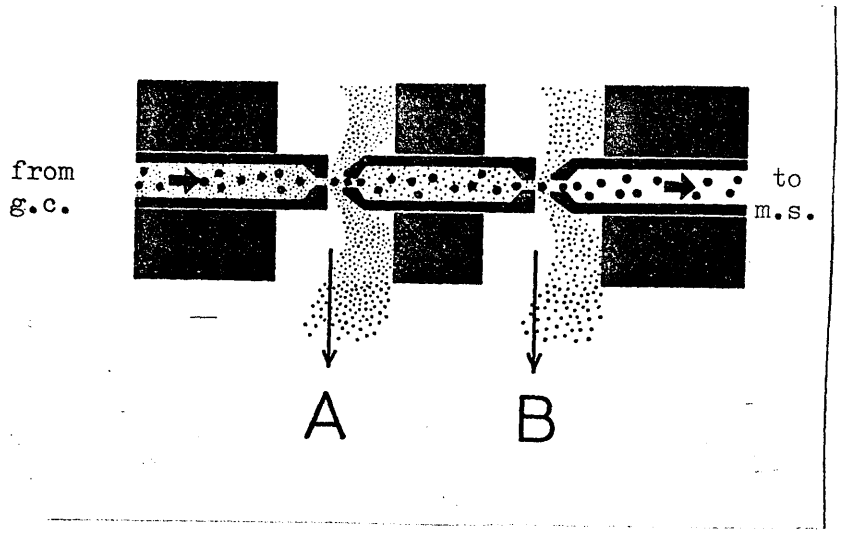

Figure 1.6. Schematic of two stage Becker-Ryhage jet-orifice separator. Connection to rotary pump (A) at 7.5 x 10^{-3} torr and connection to oil diffusion pump (B) at 2 x 10^{-4} torr.

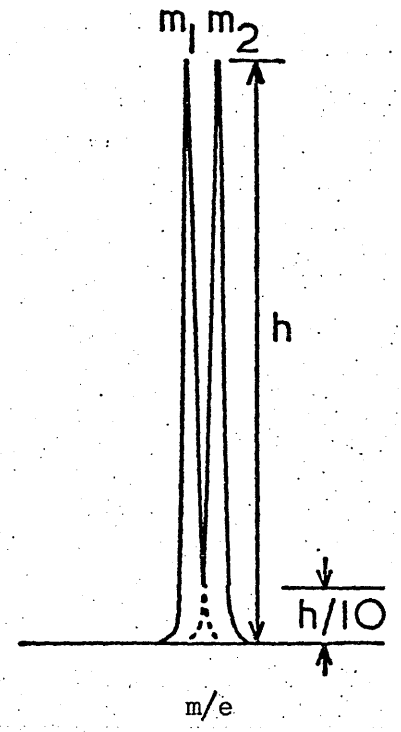

Figure 1.7. Simulated mass peaks demonstrating a mass spectral resolution of $m_1/(m_1-m_2)$.

sensitivity of the sample of decomposition are frequently amenable to g.c.-m.s. The gas chromatograph is the most convenient inlet system for routine sampling: single components are injected under conditions such that they proceed rapidly to the mass spectrometer.

However, there is a difficulty in the ideal marriage of techniques. The gas chromatograph has an outlet pressure under normal circumstances of 1 atm , whereas the analyser pressure of the mass spectrometer is normally at least 10^{-5}torr. There is also the unavoidable dilution of the sample vapour by carrier gas. If the carrier gas flow rate through the gas chromatograph is 30ml/min, a 1μg sample (MW300) emerging from the column over a period of 10sec would be present in the gas stream to the extent of 2×10^{-5} per cent (v/v). A number of molecular separator devices have been conceived as means of effecting the pressure drop and enriching the organic components of the gas stream entering the mass spectrometer. One such device is the Becker-Ryhage jet-orifice molecular separator shown in Figure 1.6. This device and the others in common use depend on the different physical properties of the carrier gas and sample to obtain the pressure drop and sample enrichment.

The operational parameters used to evaluate the performance of separators are the separation factor and the yield or efficiency.[52] The separation factor N, also often called the enrichment factor, is defined as the ratio of the sample concentration in the carrier gas entering the mass spectrometer to the sample concentration in the carrier gas coming out of the chromatographic column. Thus,

$$N = c_{MS}/c_{GC} \text{ or } N = (P_s/P_{cg})MS/(P_s/P_{cg})GC \qquad \text{(eq. 1.11.)}$$

where c_{MS} and c_{GC} are the respective sample concentrations and P_s and P_{cg} are the partial pressures of the sample and carrier gas. The efficiency of yield Y is the most important factor in the evaluation of the performance of the system. Efficiency is very simply defined as the per cent of the total sample that enters the mass spectrometer. Thus,

$$Y = Q_{MS}/Q_{CG} \times 100\% \qquad \text{(eq. 1.12.)}$$

where Q_{GC} and Q_{MS} are the quantity of sample that leaves the chromatograph and the quantity that enters the mass spectrometer.

Although the definition of yield is independent of any separation

process, it is algebraically related to the separation factor by the equation,

$$N = Y/100 \times V_{GC}/V_{MS} \qquad \text{(eq. 1.13.)}$$

where V_{GC} and V_{MS} are the carrier gas volumes measured at 760torr delivered from the gas chromatograph and to the mass spectrometer. From the last equation it is clear that the maximum theoretical value for N occurs with a 100% yield and is inversely proportional to the fraction of total carrier gas volume that enters the mass spectrometer. A 100% yield is possible only with no separator device as interface and with the hydrogen/silver-palladium separator.[62,63] In these cases N may be very high. For all other separator designs some sample is lost in the separation process. The separator designs in common use for g.c.-m.s. interface are summarised in Table 1.3. Several of these will be examined in greater detail in Section 2.3.1.

Several additional features are necessary for the mass spectrometer used for g.c.-m.s. A mass spectrum characterises a molecule not only by the masses of the molecular and fragment ions but also by their relative abundances. In batch and direct inlet operation the pressure of sample is essentially constant during scanning. However,a gas chromatogram is a profile of changing sample concentration with time. If the time required for scanning is comparable to the duration of the emerging peak, significant distortion in the true relative peak heights may occur.[69] To avoid this the mass spectrometer must be scanned rapidly - typically from m/e 50 to m/e 500 in less than 5sec. This requires a special magnet current supply and scan control circuitry. The electron multiplier is the only detection device with sufficiently rapid response for this purpose. The signal is amplified by a wide band d.c. amplifier with a frequency of 10^3-10^4Hz. Similarly the oscillographic or galvanometer display of the mass scan must be of 1000Hz band width. Under these conditions good resolution of the mass spectrum must be maintained. A useful resolution of 500 (10% valley definition)* is the minimum suitable for application to the analysis of steroids.

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* Mass spectral resolution here is defined as $R = M/\Delta m$, where M is the mass of the first peak of a mass doublet and Δm the difference the masses of the two peaks. The jargon of 10 % indicates the two recorded peaks are of approximately equal intensity and separated by a valley of 10% of the peak height. See Figure 1.7.

Table 1.3. Classiciation of commonly used molecular separators[2]

Operating principle	Construction	Designers	Reference
Effusion separators	Sintered glass frit	Watson-Biemann, Markey Copet-Evans	53,54,55
	Stainless steel sinter	Krueger-McCloskey	56
	Porous silver frit	Cree	57
	Porous silver frit	Blumer	58
	Variable conductance slit	Brunnee-Bulteman-Kappus	59
	Porous silver microseparator	Grayson-Wolf	60,61
Semi-permeable membrane			
(a) gas removal through membrane	Teflon separator	Lipsky-McMurray-Horvath	62
	Hydrogen-palladium separator	Simmonds-Shoemake-Lovelock	63
	Hydrogen-palladium separator	Lucero-Haley	64
(b) sample passage through membrane	Silicone polymer	Llewellyn-Littlejohn	65
	Silicone polymer	Black-Flath-Teranishi	66
Jet Orifice	Stainless steel jet	Ryhage-Stenhagen	67
	Glass jet	Story	68

Several techniques may be employed to obtain registration of the gas chromatogram during g.c.-m.s. analysis. This record is used by the operator to select the appropriate time for scanning. The column effluent from the gas chromatograph may be split and a proportion sent to an external detector. A flame ionization detector is most often used and the response may be designed to follow the ionization in the ion source or to precede it slightly.[70] Most commonly the concentration of sample in the source is monitored directly as the unresolved ion current. A collector plate at some point between the accelerating sector and the magnet intercepts a small portion of the beam. With this device, the energy of the electron beam must be below the ionization potential of the carrier gas to prevent the carrier gas ions from swamping sample ionization. The electron energy may be increased to the desired value for the mass spectrum at the moment of scanning. This signal is amplified and appears as a chromatogram which for practical purposes is identical to that of a flame detector.[71] A mass discriminating ionization record may be obtained directly from a quadrupole mass filter[72] or reconstructed by computer from the accumulated full scans of a g.c.-m.s. run in which continuous repetitive scanning has been used.[73]

The foregoing has had as its focus one of the most common instruments employed for g.c.-m.s.: low resolution magnetic sector type with electron-impact ionisation source. This is only one of a number of useful designs. Mass analysers useful for g.c.-m.s. are: double focusing in Mattauch-Herzog[74] and Nier-Johnson[75] configuration, time-of-flight,[76] quadrupole,[77] monopole and dodecapole.[78]

Certain limitations are imposed on the operation of the gas chromatograph in choices of stationary phase and operating temperature are governed by the stability of the stationary phase. Column bleed results in "background" ions which may hinder interpretation of spectra and limit the sensitivity in trace analysis. Column bleed may be minimised by careful conditioning at or above the analysis temperature prior to use. The nature and flow rate of carrier gas are determined by the interface.

1.4.2. Focused ion techniques. With substantial prior knowledge of the sample, the analyst may use only a few peaks in the mass spectrum in the final data interpretation. If the analogue signal for these specified m/e values is continuously monitored, it

is possible to obtain a selected ion chromatogram (mass fragmentogram). Thus the g.c.-m.s. instrument may act as a flexible and highly specific chromatographic detector. One approach consists of cyclical, rapid scanning over a limited mass range containing the ions of interest.[79] A second procedure is to arrange two or more adjacent detectors which at fixed field strength collect the ions of interest.[80] A third practice involves the selection of a single specific ion by the appropriate choice of accelerating voltage and magnet current. This method has been used to detect lead alkyls in petrol.[81] Since its introduction the technique has been justifiably popular for the qualitative and quantitative detection of known compounds. The information of the total mass spectrum may be lost, however, this is not a serious handicap when one or several characteristic ions serve for identification.

Where the sample concentration is relatively high, specific information may be obtained by continuous repetitively recorded mass spectra, but focused ion methods may offer considerable advantages in clarity of presentation and the elimination of laborious data reduction. Where compounds, present in low concentrations, can be characterised by one or a few prominent ions, the technique offers important advantages in sensitivity. The time during which each ion is in focus on the detector is substantially longer than in a full scan, so that a correspondingly larger ion current is produced. This coupled with multiplier gain of 10^6-10^8, leads to very high sensitivity. The chief practical limitations arise from the interference by ions from other components of the sample and from stationary phase bleed; also from random signals generated by the amplifier/multiplier system. The gain in sensitivity over conventional detection methods may be as much as 10^2 to 10^3.[71,82]

Of broader general applicability than the 'single ion monitoring' is the accelerating voltage alternator (AVA) of Sweeley *et al.*[83] Here the accelerating voltage at fixed magnetic field is switched sequentially from its full value to lesser values, each of which brings into focus an ion of selected mass at the electron multiplier. Thus several ion intensities may be monitored continuously during the course of a chromatographic run. Quadrupole and time-of-flight mass spectrometers may be employed, and are well adapted for programmed ion selection by virtue of the speed of the electronic principle of

mass selection in these instruments. This technique has come to be known as "selected ion monitoring",[84] or "multiple ion detection" (MID) and has been used to study drug and drug metabolite levels at very low concentrations.[85,86,87] Stable isotope species may be used as carrier and internal standards and have allowed the isolation and quantification of specific compounds at sub-nanogram levels. Prostaglandins,[88,89,90] insect juvenile hormone[91] and halucinatory drugs[92] are among the variety of compounds analysed by this method. Progress in the field has been reviewed and its impact on the investigation of drug metabolism has been assessed recently by Gordon and Frigerio[93] and by Millard.[94] Several systems have been described for full control and data processing by computer for MID. These have been summarized by Mellon[95]

Computer acquisition and reduction of data facilitate the application of g.c.-m.s. Continuous monitoring of the chromatographic effluent by repetitive scanning on a 2-4sec cycle generally serves to record the significant information. Automatic processing of the data, during or after each run, frees the analyst from the drudgery of manual calibration, measurement and normalisation of the mass spectra; useful manipulations of the data generated are also possible, as exemplified by the following. The total ionisation current chromatogram may be reconstructed by summing the intensities of ions recorded in each scan, with the particularly useful option of disregarding ions of low mass. The intensity of any ion through the course of the analysis may be displayed as a selected ion chromatogram. Any number of relevant ion chromatograms may be obtained, not being restricted to the number of channels in an instrumental MID system, but as noted above, the sensitivity is limited by the relatively low ion currents obtaining in full scanning.

1.4.3. Chemical transformations in transitu. Numerous techniques are available for the modification of samples in the chromatographic system; before, on, or just after the chromatographic column.[96,97] Several have been employed in dynamic combination with a mass spectrometer. Hydroxylic, enolic and other readily exchangeable hydrogen atoms may be selectively replaced by deuterium during gas chromatographic separations.[98,99] The stationary phase is coated with a suitable catalyst, usually barium hydroxide, and the column is "saturated" with D_2O or MeOD

just prior to the analysis. The use of deuterium may be very useful in the investigation of mass spectral fragmentation and this technique has been used to investigate the mass spectra of some steroidal ketones and their derivatives.[100]

Reaction gas chromatography has been widely used for the identification of small quantities of materials by their behaviour after hydrogenation, hydrogenolysis or pyrolysis. Identification of the resulting materials has been by retention index comparisons, by pattern recognition in the resulting pyrogram, and by gas chromatography-mass spectrometry. Henneberg and Schomberg[101] have used hydrogenation in a micro-reactor before and after g.c. separation in combination with g.c.-m.s. for the elucidation of the composition of complex mixtures of olefins. Pyrolysis g.c.-m.s. has been recently applied to the analysis of triglycerides[102] and three acyclic monoterpenoids in the oil of Ledum palustre have been identified by "carbon skeleton gas chromatography" (catalytic stripping of all functional groups and complete saturation) together with g.c.-m.s.[103]

1.4.4. Combined instrumental techniques. Many instrumental techniques have been used in direct combination with gas chromatography.[97] However, only infrared spectroscopy has been used in a tripartite combination with gas chromatography and mass spectrometry.[104] The problems of scanning of both instruments during the short emergence of the g.c. peak is normally solved by stopped flow techniques.[105]

1.4.5. Derivatives for g.c.-m.s. There are two general ways in which derivatives may be employed to affect mass spectra. The derivative may, without altering the fragmentation mode, introduce mass changes that facilitate interpretation. This may be accomplished by isotopic or other minor structural substitution. Alternatively, a particular mode of fragmentation may be promoted either to assist structural elucidation or to produce specific ions for analytical purposes.

(a) Production of mass shifts. The simplest mass shifts are in the molecular ion, for which any effect of a change in molecular mass on the fragmentation is least significant. Specific adjustment of the isotopic composition to alter the patterns of molecular or fragment ions may be informative. For example, the replacement of exchangeable hydrogens with

deuterium is useful. A novel technique for the recognition of metabolites in pharmacological and biochemical investigations involves the mass spectrometric analysis of partially labelled drugs or other precursors. Typically one or more isotopes are present in such proportions that the labelled centre produces conspicuous "twin ions" or other well defined patterns of ions. Metabolites unaltered at the site of labelling are readily detected by g.c.-m.s. inasmuch as their mass spectra preserve the characteristic patterns.[106,107,108]

Another important application of isotope substitution in g.c.-m.s. is to provide an internal standard in the quantitative analysis of the corresponding unlabelled parent compound (or vice versa). The distinction between the two compounds, easily assessed from the mass spectra, in usually obtained with several deuterium atoms at sites in the molecule not seriously affected by fragmentation. The difference in retention time is generally small, and the chromatographic properties of "unknown" and reference compounds, being very similar, simplify extraction and work-up procedures and increase the reliability of the analysis. Large amounts of labelled material may be added to the sample containing the unlabelled parent prior to analysis. Adsorption effects in the gas chromatography at low sample levels are thus minimised by the large excess of "carrier" co-chromatographing with the sample.[88]

Functional group derivatives produce generally larger mass shifts in the molecular ion and may substantially alter fragmentation. Implicit in the application of functional group derivatives for g.c.-m.s. analysis is the association of characteristic retention changes with derivative formation. These changes depend on the chromatographic conditions employed and are interpreted in structural terms in conjunction with concomitant changes in mass spectra.

(b) Modification of fragmentation. Derivatives are frequently effective in yielding molecular ions where such ions appear in low abundance (or not at all) in the parent compounds. Ions formed by simple losses of radicals may also be indicative of the molecular weight. For example, the TMS ether derivatives of alcohols sometimes produce more prominent molecular ions than their parent compounds: the usually prominent $(M-90)^{+\cdot}$ ion, resulting from the elimination of trimethylsilanol, gives an additional indirect indication of

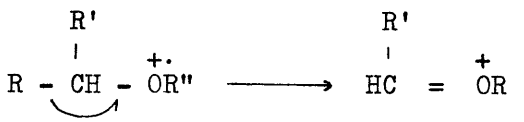
Table 1.4. A partial list of functional derivatives useful for gas chromatographic and g.c.-m.s. analysis

Functional group	Reagent	Derivative	Mass increment (Δm)	Reference
R-OH	*Hexamethyldisilazane (HMDS), trimethyl-chlorosilane (TMCS), etc.	R-O-Si$(CH_3)_3$	72	109
R-C=O | R	Methyl hydroxylamine	O-methyloxime	29	110,111
	HMDS and Me_3SiBr	Enol TMS ether	72	112
RCOOH	CH_2N_2	RCOOMe	14	113
	*Silylating reagents	TMS esters	72	114
R_1NH_2/R_1R_2NH	Trifluoroacetic anhydride	R_1-NCOCF$_3$ / R_2	96	115

* The variety of silylating reagents available and their application are treated in a monograph by Pierce.[110]

the molecular weight.

Derivatives are also of great importance for their directive effects on mass spectrometric fragmentation. A useful example is the enhancement of α-cleavage in methyl and TMS ether derivatives of secondary alcohols (Scheme 1.2.):



Scheme 1.2.

This type of α-cleavage is also involved in the highly characteristic fragmentation of TMS ethers of Δ^5-3-hydroxy-steroids (see Section 2.3.8.).

(c) Reagents for derivative formation. A variety of functional group derivatives are suitable for g.c.-m.s. analysis, each associated with an increase in molecular weight. Table 1.4. provides a small sample of these for common functional groups with the associated mass increments. While certain derivatives may be used with a wide range of individual functional groups some are capable of selective application. For example, a number of trimethylsilylating agents exhibit reactivities different enough to discriminate between particular types of hydroxylic groups in steroids. However, under forcing conditions, enol ethers may be obtained, even of the highly hindered 11-oxosteroids. While diazomethane is potentially reactive with many compounds, in practice it is largely selective for methylation of carboxylic acids, since its numerous other reactions are generally much slower. In contrast, there are reagents which normally react with only a small range of functional groups.

A valuable group of reagents comprises those that are suitable for forming cyclic derivatives of compounds possessing bi- or multifunctional reactivity. This kind of process is of great importance because its selectivity depends on the particular relative disposition and stereochemistry of the reactive groups in the substrate. The formation of a ring limits the range of possible conformations, and gives rise to distinctive chemical, chromatographic and mass spectrometric properties.

For multifunctional compounds, the use of "mixed" derivatives prepared by sequential treatment with reagents of distinctive

selectivity may be of value. Such derivatives offer the possibility of controlling both gas chromatographic and mass spectrometric properties according to the information sought. The use of isotope-labelled or homologous reagents can further assist in the recognition of structurally significant ions. While the classical connotation of the term "derivative formation" implies a simple, potentially reversible change, it is not inappropriate to consider other transformations which facilitate analyses by causing distinctive changes in chromatographic behaviour and fragmentation. The use of cholesterol oxidase to convert 3β-hydroxysteroid-5-enes into 4-en-3-ones is an example of this type.[117]

1.5. The Scope and Limitations of Combined Gas Chromatography-Mass Spectrometry

1.5.1. Scope. The impact of g.c.-m.s. as an analytical method is reflected in the number and diversity of publications dealing with its development and use. Since the appearance of early papers describing the principle, summarised in early reviews such as that of Littlewood,[118] growth has been rapid. New disciplines have adopted the technique as the instrumentation became available and its applicability came to be appreciated. The field has been intensively reviewed in the chapters on gas chromatography-mass spectrometry appearing in the Chemical Society Specialist Periodical Reports on Mass Spectrometry.[119] While not comprehensive, these offer an indication of the increasing activity: Volume 1 (1968-1970), 221 references; Volume 2 (1970-1972), 405 references and 24 books or reviews; Volume 3 (1972-1974), 592 references and 48 books or reviews; Volume 4 (1974-1976), 647 references and 52 books or reviews. Gas Chromatography-Mass Spectrometry Abstracts demonstrated a similar growth.[120] Recently a separate journal has appeared devoted exclusively to the application of mass spectrometry to biological, environmental, and medical science.[121] A substantial proportion of the applications appearing in this publication employ g.c.-m.s. Most materials which may be usefully and successfully chromatographed in the gas phase may be advantageously subjected to analysis by g.c.-m.s. These various sample types provide a useful framework for the classification of g.c.-m.s. applications.

Volatile and thermally stable compounds may be chromatographed

directly and sample vapour identified by mass spectrometry after separation. This class of materials covers a wide molecular weight range and includes, for example, volatile acyclic and alicyclic hydrocarbons of industrial, geological, and biological origin. Most environmental pollutants and pesticides may be analysed directly, as well as flavour, aroma and essential oil samples. Numerous samples of biological origin are also stable for direct analysis, e.g. volatiles of biological fluids, some insect pheromones. Also included are suitable compounds from more routine synthetic and preparative organic chemistry.

A second important class are those polar, non-volatile or heat sensitive compounds which require derivatization to increase volatility and improve thermal stability in gas chromatography. Volatile derivatives offer important advantages, particularly in reducing the column temperature that must be used for g.c.-m.s. with important benefits in respect of column background in the mass spectrometer and reduction of thermal decomposition. While under favourable conditions many polar compounds may be chromatographed directly, judicious derivative formation may be useful; of course, depending on prior knowledge of the sample. For example it would be uncommon for the analysis of an essential oil to have any more extensive a conversion carried out than methyl esterification. On the other hand; for the analysis of most steroids, amino acids, carbohydrates, bile acids, etc. derivative formation is essential. Polyfunctional compounds are often protected by mixed derivatives. A judicious choice of derivatives may produce materials of molecular weight more advantageous for the analysis. In some cases the interpretation of mass spectra may be facilitated. A comprehensive survey may be obtained from recent review articles.[119,122,123]

Finally are the compounds which are so complex or involatile as to require degradation to constituent parts to be suitable for analysis. These include the large polysaccharides, polypeptides and nucleotides which figure importantly in biological function. The investigations of Biemann and co-workers of polypeptide sequencing[124] are prominent in this field. This work involves specific enzymatic degradation, and partial acid hydrolysis, separation and identification of the resulting mixture of overlapping oligopeptides. Computer techniques are then employed in the reconstruction of the original polypeptide. Recent progress in protein and carbohydrate sequence analysis by

mass spectrometry and g.c.-m.s. has been summarised by Norris and Dell.[125]

1.5.2. Limitations. Gas chromatography-mass spectrometry as a combined technique is subject to the limitations of the constituent methods. Absorption and decomposition of sensitive materials on the column, and insufficient chromatographic resolution are the major difficulties associated with gas chromatography. Stable isotope techniques have found use in dealing with absorption/decomposition in g.c.-m.s.

Work with the object of improved separation by the development and use of open tubular columns of higher efficiency will be discussed in detail in Section 2. However, it is interesting to note that the direct analysis of complex mixtures; such as the 'urinary steroid profile', which offered much of the impetus for workers developing high resolution glass open tubular columns[126-130] is usefully complemented by investigations in which rapid and informative preliminary fractionation is carried out by Sephadex gel chromatography.[131,132]

Limitations also arise in distinguishing or making structural assignments of some stereoisomeric materials by mass spectrometry. For example, some of these difficulties have been re-emphasized by Mulheirn[133,134] in relation to the study of isomeric steranes of geochemical origin. However, good chromatographic technique may serve to distinguish isomers for which mass spectra are non-specific. For example, enantiomeric compounds such as secondary alcohols and amines have been resolved gas chromatographically by the use of chiral derivatives.[135]

The analysis of the bitumen extracted from the Green River Formation Oil Shale aptly demonstrates the power of combined gas chromatography-mass spectrometry and also its limitations. The discovery in this and other sediments and fossil fuels from various geological ages of organic compounds with skeletal features closely related to compounds produced by contemporary living organisms is a potent argument for the biological origin of these organic deposits. Among these 'biological markers,[136] are intact and modified paraffins, isoprenoids, alcohols, amino-acids, ketones, carboxylic acids, steroidal carboxylic acids, terpanes, steranes, and porphyrins.[137] The highly characteristic mass spectra of the many isoprenoid paraffins, steranes, terpanes and carotanes found in crude oils and shales make these compounds

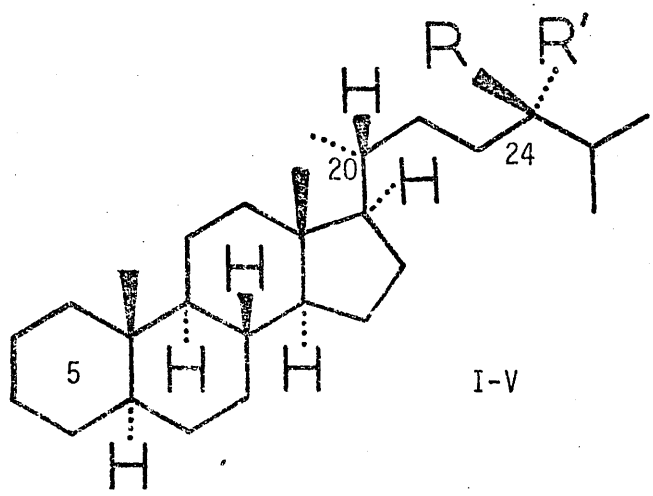


Figure 1.8. Structural formulae of steranes isolated from biological and geological sources. The stereochemistry of the perhydro-1,2-cyclopentano phenanthrene ring system and R stereochemistry at C_{20} in the side-chain in 5α-and 5β-configuration is the common biogenetic structure. Only the 5α-epimer is shown. I: R = R' = H; 5α-cholestane. II: R = methyl group; R' = H; 5α-campestane (24R). III: R = H; R' = methyl group; 5α-ergostane (24S). IV: R = ethyl group; R' = H; 5α-stigmastane (24R). V: R = H; R' = ethyl group; 5α-poriferastane (24S). Stereochemistry and nomenclature of the steroid skeleton is discussed in detail in Section 3.1.1.

particularly amenable to mass spectral analysis. Combined with gas chromatography the rapid separation and characterisation of these complex mixtures may be undertaken. However, the initial mixture of hydrocarbons and polar compounds are generally too complex to allow direct investigation of the total sample. Subfractionation by extraction and distillation, thiourea adduction,[138] digestion over molecular sieve,[139] alumina column adsorption,[140] or liquid gel-filtration[141] are prerequisite for analysis to reduce the complexity of the mixture to be analysed. The limitations imposed by mixture complexity are further reduced by the use of high resolution open tubular columns for the analysis. Among the compounds in the branched cyclic hydrocarbon extract of Green River shale are large proportions of C_{16}, C_{18}, C_{19} (pristane) and C_{20} (phytane) isoprenoid alkanes, 5α-pregnane, 5α- and 5β-isomers of C_{27}, C_{28}, and C_{29} tetracyclic steranes, gammacerane and hopane ($C_{30}H_{52}$) and several pentacyclic triterpenoic alkanes. These identifications have been reviewed by Maxwell *et al.*[142] Gas chromatography-mass spectrometry is well suited for such an analysis. However, some ambiguities in respect of the identification of C_{24} alkylated steranes arise. Figure 1.8. shows the structural formulae of these materials. Diastereomers of this type (24R- and 24S alkyl cholestane derivatives) are indistinguishable by infrared spectroscopy, mass spectrometry, gas chromatography, or by chiral alkylated Sephadex gel liquid chromatography.[143,144] The problem was attacked by the separation and isolation by automatic, high resolution, preparative gas chromatography of the C_{27} and C_{28} steranes in milligram quantities of high purity.[145] ^{13}C nuclear magnetic resonance spectroscopy (n.m.r.) was employed in these materials and the stereochemistry of the ring junctions in the nucleus was confirmed as the biogenic configuration.[146] These data, together with additional data from high resolution (100 or 220MHz) proton magnetic resonance (p.m.r.) spectroscopy and optical rotatory dispersion (o.r.d.)[147] are consistent with the conclusion that in addition to 5α-ergostane (24S) suggested initially,[138] there is present a significant proportion of the other C_{24} epimer 5α-campestane (24R). Such information was inaccessible by g.c.-m.s. alone. It is interesting to note that a t.l.c. method for the separation of 24-alkyl epimers of steranes has recently appeared.[148] Analysis of so complex a mixture as the Green River Shale bitumen

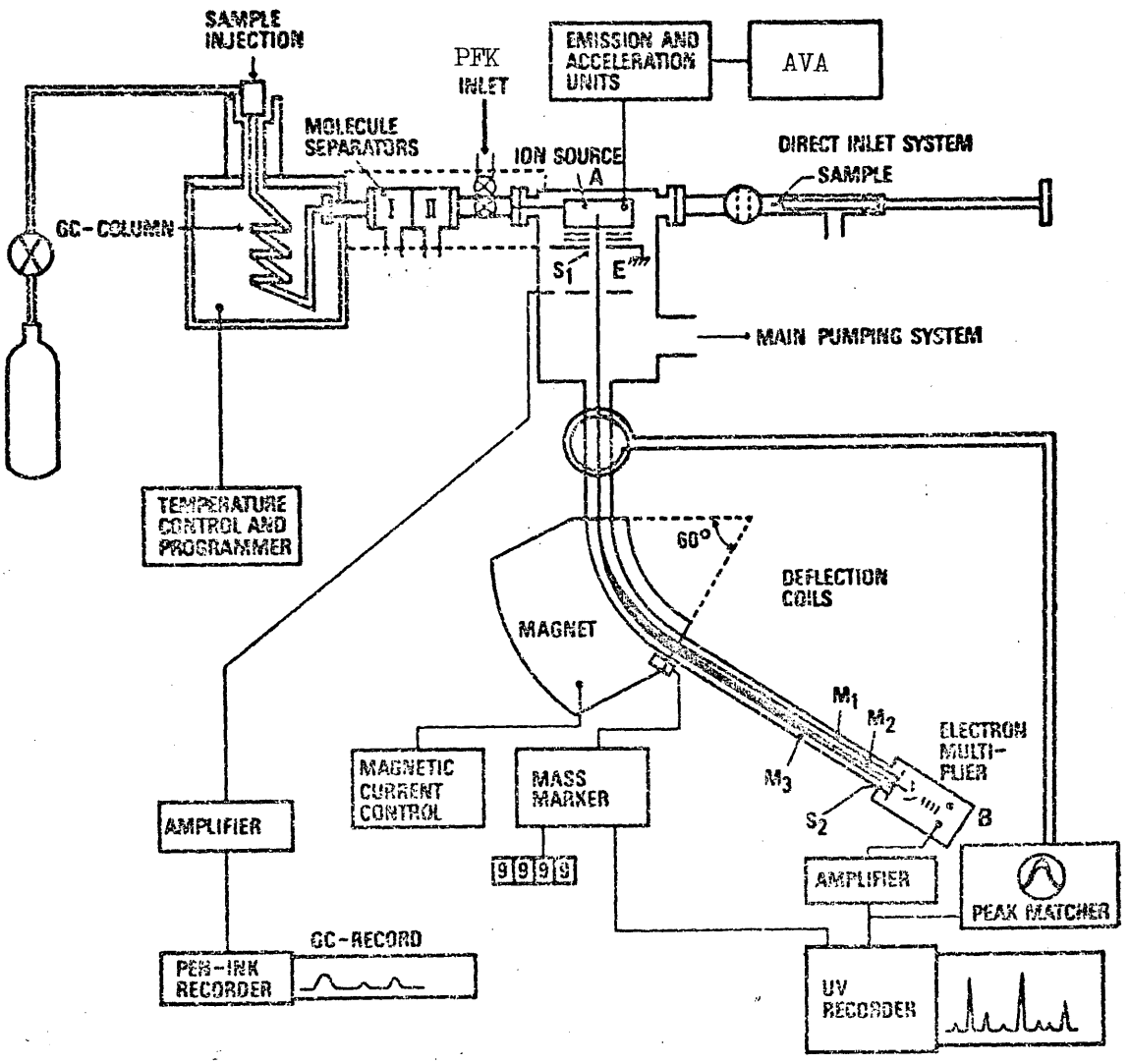


Figure 1.9. Schematic diagram of the LKB 9000 combined gas chromatograph-mass spectrometer in the G.C.-M.S. Unit, Department of Chemistry, The University, Glasgow. Diagram reproduced by permission of LKB Instruments Ltd., London.

might never have been undertaken without the availability of combined gas chromatography-mass spectrometry for its rapid, selective and sensitive analyses. None-the-less, the foregoing illustrates that for maximum impact the method must be employed within a broad mutually supporting analytical framework which takes into account the strengths and weaknesses of each constituent technique.

The following sections of this thesis relate to work carried out by the author in the period October 1971 to November 1974. The g.c.-m.s. facility in the present work is in the Department of Chemistry of the University of Glasgow and is based on an LKB 9000 gas chromatograph-mass spectrometer*. This comprises a gas chromatograph with temperature programmer, a Ryhage-type jet molecular separator and a single focusing mass spectrometer equipped with a 60^{o} sector, 20cm radius magnetic analyser and sweep generator for fast scanning of spectra (see Figure 1.9.). A rhenium filament is used to provide an ion source of the electron bombardment type. The measuring system comprises a 14-stage electron multiplier, electrometer pre-amplifier and a wide-band amplifier feeding a direct-writing ultraviolet light oscillograph. A direct probe inlet was available and a separate inlet system for the introduction of marker substances. Various gas chromatographs were employed as indicated in the text.

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* LKB-Produkter AB, Stockholm Bromma 1, Sweden.

2. GLASS OPEN TUBULAR COLUMNS IN GAS CHROMATOGRAPHY AND COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY

2.1. Column Preparation and Performance

2.1.1. Introduction. In the utilization of open tubular columns and in particular glass open tubular columns, a major source of difficulty has been the maintenance of a homogeneous thin film of stationary phase on the interior surface of the column. If the temperature of the column was raised or the polarity of the stationary phase increased, the formation of micro drops and lenses of phase on the hydrophobic wall of the column was common. Thus the useful life time of the columns, particularly those operated at high temperatures for the analysis of relatively involatile materials, were measured in hours or days.[149] Several approaches have been developed to overcome this problem. Each technique seeks to alter the nature of the surface wetted by the stationary phase and thus improve its stability.

(a) Surface corrosion. The stability of the thin film of stationary phase on finely particulate silicaceous column packings is the result in part of their large, irregular surface area. Techniques were devised to increase the specific area of the interior of the open tubular column by etching the surface. Alkaline etching in aqueous solution[150,151] has proved generally unsatisfactory for columns intended for biochemical analyses because of the catalytically active and polar nature of the porous layer produced and the resulting absorption and tailing.[152] More satisfactory results have been obtained by gas phase etching with dry hydrogen chloride[153,154] and dry hydrogen fluoride[154] as well as HF produced pyrolytically *in situ* from methyl trifluoro-chloroethyl ether sealed within the glass spiral heated at 300°C.[155] The etching of soft ("soda-lime") glass columns has proved useful for the coating of polar phases where the wetting characteristic of the surface is particularly important. Columns of polar phases useful in steroid analysis have been prepared in this manner.[127,156] Franken *et al.* have reported a general method for the preparation of such columns for a number of applications.[157]

(b) Carbonization. With the observation very early that graphite might be an excellent support for liquid phase in gas liquid chromatography,[158] and work evaluating the contact angle of organic liquids on carbonized glass,[159] techniques for the coating

the interior of open tubular columns with a uniform thin film of carbon have received attention. The deposition of a carbon layer by means of the pyrolysis of methylene chloride[160,161] and the coating of colloidal graphite produced by sonication in suspension in methylene chloride[162] have produced columns of reasonable quality with a variety of polar and non-polar stationary phases.

(c) Organic intermediate layers. Films of organic materials which adhere to the glass bore and are themselves wetted by the stationary phase have proven useful. A mechanically deposited layer of Bentone 34, a chemically modified benonite,[163] and layers of polybutadiene and polytrifluorochloroethylene,[161] polymerized from the gaseous monomer have been employed with good effect. The main limitation is the softening of the layers with increase in temperature.

(d) Oriented monolayers. Many monofunctional compounds form oriented monolayers on clean glass surfaces in which the polar portion of the molecule interacts with the surface groups and the remainder of the molecule is extended outwards. It has been observed that the ideal surface treatment in these circumstances involves selected substances which consist in alpha-omega difunctional molecules in which one group bonds to the surface of the glass and the other is matched to interact with the stationary phase.[152] The application of surfactants such as Gas-Quat L (trioctadecylmethylammonium chloride)[164] or BTPPC (benzyltriphenylphosphonium chloride)[165] is desirable from the standpoint of surface deactivation as well as improving surface-stationary phase compatibility.[161] Particularly encouraging results have been obtained employing these reagents in conjunction with surface deactivation by silylation to prepare columns of apolar phases for use in steroid analysis.[126]

(e) Chemical modification of silanol groups. Glass deactivation is paramount in the analysis of sensitive compounds by gas liquid chromatography. Replacement of the active hydrogens of surface silanol groups with silyl groups by silylating reagents in solutions or in gas phase[116,166] is a standard in the preparation of column packings and apparatus, as well as in their maintenance in use.[167] The basic trimethylsilyl structure of these reagents may be elaborated to match the stationary phase and improve stability of the phase film. A number of alkyl and haloalkyl silylating reagents have been employed with success with apolar

phases.[168] The more difficult problem of more polar phases has been approached by the oxidation in situ of allyl silane.[169] Further to the "tailoring" of the bore of glass open tubular columns, silylation has been carried out with dimethylchloro-4-(4-chloromethyl-phenyl)butylsilane. The benzylic chlorine thus bonded to the surface is replaced with various polar substituents selected to suit the particular polar phase.[170]

An additional possibility exists for the covalent bonding of polymeric material as stationary phase to the interior of the column in the same manner as that employed in the preparation of packings for gas-liquid and liquid-liquid chromatography.[171,172,173] This concept has been applied to glass open tubular columns by Madani and co-workers.[174] A polymeric mixture, prepared by hydrolysis of dimethyldichlorosilane and coated on a pre-etched glass column, is covalently bound to the hydroxyl groups of the glass matrix by a base catalysed reaction. These columns, of a polarity similar to methylsiloxane phases (e.g. OV-1), are useful for separations of complex steroid mixtures and the method may extend to polymers of a more polar nature. The success here indicates the possibility of columns with very low bleed particularly suitable for g.c.-m.s. applications.

(f) Porous layer open tubular (PLOT) columns. Golay observed in 1960 that an advantage could be obtained by coating a thin layer of support on the interior of the conventional wall coated open tubular (WCOT) columns, thus providing an increase in surface area and a corresponding reduction in the thickness of the film as well as stabilizing the film of stationary phase.[175] Columns utilizing this concept were constructed with such diverse substances as Al_2O_3, $SiO_2 \cdot nH_2O$ and finely divided maize flour.[161,176] However, the most successful material for support was finely divided diatomaceous earth, normally utilized as column packing. This material has been coated on open tubular glass and metal spirals to produce the now familiar support coated open tubular (SCOT) columns of Ettre[177,178,179] and other workers.[180,181]

The appearance in commercial quantities of a finely divided silylated fumed silicon dioxide, Silanox 101*, prompted work which showed that stable colloidal suspensions in solutions of apolar liquid phases readily adhered to the interior of metal[182,183] and

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* Silanox 101, 6-10 diameter: Cabot Corporation, Billericia, Mass., U.S.A.

glass[128,184] columns. Silanox 101 may also be used with more polar phases[185,186] and these porous layer columns of various polarities have found application in steroid analysis. These will be discussed in more detail in later sections. An additional elaboration of the principle involves coating of an unsilanized form of this fumed silicon dioxide, Cab-O-Sil*, onto the untreated interior of the column and cross linking the material with dimethyldichlorosilane, which simultaneously cross-links the porous material and deactivates the interior of the columns to produce an inert, stable and re-usable support for apolar stationary phases.[187]

(g) Whisker walled open tubular (WWOT) columns. An extraordinary recent development is the appearance of a method for the production of silicone dioxide "whiskers" on the interior of glass columns.[188] Production of a dense layer of villi-like structures, several microns in diameter and several tens of microns in length, is effected in the vapour phase by a fluoro-ether (e.g. 2-chloro-1,-1,2-trifluoroethyl methyl ether) sealed in a glass spiral at an elevated temperature (300-500°C). It has been shown that, with careful deactivation and coating, columns of this type are comparable or superior to other types.

2.1.2. Column preparation. The columns prepared for use in this work are essentially those of German and Horning.[128] In this procedure a two-stage dynamic coating routine is employed in which the finely divided Silanox is coated in suspension in a solution of chloroform, 0.5 per cent stationary phase and the surfactant, BTPPC. Following drying, additional stationary phase is applied by the same method in a 2 per cent solution in isooctane. German and Horning in their pioneering paper pointed out that the Silanox had no special attachment to the column wall and as a result of the two stage coating procedure, the Silanox may not be evenly distributed through the layer. Indeed, there is evidence to suggest that the Silanox in fact floats at the surface of the phase layer.[190] The authors state only that in their opinion the desirable properties of the columns lie with the Silanox-liquid phase combination and the effect the particulate material exercises on the "wettability" of the liquid phase. This calls into question the proper classification for these columns. In preparation they resemble SCOT columns but it is questionable

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* Cab-O-Sil, Packard, Downers Grove, Ill., U.S.A.

whether the Silanox serves any "support" role. Though this uncertainty is unresolved, it is felt that the designation as porous layer open tubular (PLOT) is useful. This is an opinion shared by other workers.[182,189]

(a) Drawing of glass columns. A standard 1.5m length of 7mm medium wall pyrex glass tubing* was washed thoroughly with detergent, rinsed and allowed to stand in 5 per cent aqueous potassium hydroxide. This blank was then washed to neutrality with water and with successive 200ml portions of AnalaR methanol, chloroform and acetone, and dried under vacuum. The glass column was then drawn into a narrow bore spiral with a device constructed according to Desty.[191] In principle the machine consists of one pair of rollers driven by a synchronous motor which forces the glass blank into an electrically heated oven. A second pair of rollers continuously draws out a capillary from the molten glass. The dimensions of the resulting capillary are dictated by the ratio of the speeds of the two electric drives, in this case about 1 in 10. The capillary is driven into an electrically-heated former which bends the tube into a spiral which is accepted onto a rotating spindle. Under these conditions a spiral of 12.5cm to 13.5cm in diameter was produced whose internal diameter (i.d.) was 0.5mm and external diameter (o.d.) 0.9mm. If the drawing procedure proceeded uninterupted the blank would produce a spiral of some 120 turns, 85m in length. In practice the procedure seldom proceeded without incident and a success rate of 50 per cent for spirals over 35m was found to be normal.

(b) Column deactivation. The interior surface of the column was silanized in gas phase according to the method of Novotny et al.[166] A stream of dry N_2 was saturated with vapour of a mixture of 5:1 ratio hexamethyldisilazane (HMDS: Pierce Chemical Co., Rockford, Ill., No. 84770) and trimethylchlorosilane (TMCS: Koch-Light Laboratories Ltd., Colnbrook, Bucks., No.5501) and passed through the column spiral for a period of 30 min. at 30ml/min. The ends of the spiral were sealed by fusion and the spiral heated at 200°C for at least 72 hours (periods of heating occasionally extended to 3 weeks). The columns were stored sealed, under the same silylating reagents at room temperature until required for coating.

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* Pyrex glass tubing: O.D. 7mm ± 0.4mm, wall 1.5mm ± 0.2mm, James A. Jobling, Stone, Staffordshire, ST15 0GB.

(c) Column coating. The uncoated column was opened and the excess reagents were removed by rinsing the column with 5ml of AnalaR methanol. The column was dried by passing dry N_2 through at approximately 30ml/min and a second silanization step performed. A 5 per cent (volume/volume) of dimethyldichlorosilane (DMCS: Hopkin and Williams, Chadwell Heath, Essex, No. 37795) in AnalaR toluene was passed through the column as a plug of approximately 25 per cent of the column length which traversed the column in a period of approximately 30 minutes. A plug of AnalaR toluene followed to remove excess reagent and the column was then rinsed with 5ml of AnalaR methanol and dried in a stream of dry N_2 for two hours. The apparatus employed for the coating of both initial and liquid phase layers, as well as the percolation of all other solvents and solutions required for column preparation, is essentially that of Merle d'Aubigne et al.,[192] using a commercially available version of their apparatus.* The coating of the Silanox layer proceeds with a suspension of 2g of Silanox 101 in 100ml of AnalaR chloroform containing 0.2g of BTPPC (R.N. Emanuel Ltd., Wembley, England, No.B32807) and 0.5g of OV-1 (Field Instruments, Richmond, Surrey). The high density of the solvent and the use of surfactant produced a suspension stable enough not to require agitation in use. However, after any period of storage it was found necessary to resuspend the Silanox by a 20min period of sonication in order to avoid plugging the column during coating. The column was attached to the coating resevoir by means of a 1/16" Swagelok coupling equipped with PTFE ferrule (Chemical Research Services, Inc., Addison, Illinois, No.SF 100). Similarly, a 1/16" Swagelok union with PTFE ferrules was used to connect the column to a 20m "pigtail" column of the same diameter (a failed or retired column), thus stabilizing the velocity of the coating plug as it emerges from the column. The column was wetted by a short plug of chloroform immediately followed by a plug of the Silanox suspension. The plug was 40 per cent of the column length, propelled by dry nitrogen from a pressure regulator at a rate of 5cm/sec. A plug length somewhat longer than that quoted by the original authors (25 per cent) was found desirable to counteract the tendency of the plug to accelerate during the coating, reducing the homogeneity of the phase film. After the plug was expelled, the nitrogen pressure was increased and the

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* Scientific Glass Engineering Pty, Ltd., 657 North Circular Road, London, NW2 7AY, Cat.no. CR2.

column dried at room temperature for 2 hours. It was found that the pressure of the drying stream must not be so high as to cause rippling in the film created by turbulent gas flow within the column.

The dynamic coating of the liquid phase layer proceeded with the same arrangement, using a solution prepared from 100ml of isooctane (BDH Chemicals ltd., Poole, England, No.30518) and 2g OV-1. A plug of 40 per cent of the column length was forced through at a rate of 2cm/sec. Immediately the plug left the column, the nitrogen pressure was increased, care taken to avoid turbulent flow, and the flow maintained overnight. The columns were conditioned either installed in the gas chromatograph or in a separate conditioning oven. Gas flow was 15ml/min, and the oven temperature increased at $1^{\circ}C$/min to $200^{\circ}C$; this temperature was maintained for at least 6 hours. Gas flow was then reduced to 2ml/min and the temperature increased to $300^{\circ}C$ at $1^{\circ}C$/min and maintained at that temperature for 4 hours. A number of turns at the beginning and the end of the spiral thus prepared and conditioned were broken off and discarded and the column was installed in one of two gas chromatographs modified for its use. (See: Section 2.2.). Any subsequent coatings were performed in the identical fashion.

2.1.3. Column Performance. Table 2.1. summarizes the columns prepared and tested. The columns are shown in essentially chronological order of their preparation. The notes on "coating" in that table reflect the elaboration of the procedure of German and Horning to include additional gas-phase silylation and additional coating of stationary phase after initial coating. Helium was adopted as carrier gas for routine chromatography for the enhancement in efficiency which it offers, as well as shorter analysis times produced by higher flow rates.

(a) Gas chromatographic measurements. For evaluation the columns were installed in a Pye Model 104 gas chromatograph in a support cage and with dry injector. Additional gas was added to the column effluent just prior to its entering the flame ionization detector (FID). The number of theoretical plates (N) was determined by the relation:

$$N = 5.54 \left(t_r / w_{\frac{1}{2}}\right)^2 \qquad \text{(eq. 2.1.)}$$

Table 2.1. Summary of column preparation and performance.

No.	Column	Coating	CG/rate (ml/min)	Theoretical plates (N)	HETP ($\times 10^{-3}$m)	Performance factor (Theoretical plates/m)	Separation no. nC_{24}-nC_{28}(z)	Comments
M1	0.5mm x 25m	G & H*	N_2/1	17,500	1.42	700	-	Low capacity observed
F1	0.35mm x 35m	G & H	N_2/1	26,900	1.30	768	-	
M3	0.5mm x 35m	G & H	N_2/1	30,625	1.14	875	32.2	
M10	0.5mm x 35m	200°Silyl** + G & H	N_2/1 He/5	29,066 36,447	1.20 0.96	830 1041	20.6 -	Determine to use He routinely as carrier gas
M6	0.5mm x 70m	200°Silyl+G&H	He/2	72,777	0.96	1039	59.3	
M2	0.5mm x 50m	200°Silyl+G&H 2nd G&H	He/4 "	31,900 84,904	1.56 0.58	1301 1698	- 62.2	Column used extensively for 5 months, gradual loss of efficiency, retired at n = 55,000. 2nd coating to no effect.
M8	0.5mm x 50m	200°Silyl+G&H 2nd G&H	He/5 "	67,034 67,340	0.74 0.74	1340 1340	- 44.5	
M12	0.5mm x 50m	200°Silyl+G&H 2nd G&H***	He/5 "	43,488 61,369	1.15 0.81	869 1227	- 47.5	Improved by 2nd coat.
M14	0.5,, x 50m	200°Silyl+G&H 2nd G&H***	He/5 "	40,902 53,115	1.31 0.94	760 1063	- 47.8	"

* Coating procedure according to A.L. German and E.C. Horning, J. Chromatogr. Sci., 11 (1973) 76.

** Gas phase silylation according to M. Novotny et al., J. Chromatogr. Sci., 8 (1970) 390.

*** Silanox 101 layer omitted in subsequent coating procedures.

Note:
A useful but not regularly evaluated column characteristic was the partition (capacity) ratio (k') measured by

$$k' = tr/td$$

where tr is the corrected retention time and td is the column dead time. For n-tetracosane at 250°C k'=5-10 for the later columns.

at a carrier gas flow which was determined to produce maximum efficiency for the test substance. In Equation 2.1., t_r is the retention time of the test substance and $w_{\frac{1}{2}}$ the peak width of the test substance at half height. The test substance was routinely n-octacosane ($n\text{-}C_{28}H_{58}$). 0.5µg was injected at column temperatures selected to produce convenient analysis times (220°C-270°C) for the length of column. Height equivalent to a theoretical plate (HETP) was calculated by:

$$HETP = L/N \qquad \text{(eq. 2.2.)}$$

where L is the length of the column in centimetres. Similarly performance factor (n) was calculated as the reciprocal of Equation 2.2. and determined the average number of theoretical plates in a metre of column length.

A summary parameter of column performance is given in the separation number (z) of Kaiser.[193] This quantity provides an estimation of the number of peaks which can be satisfactorily separated between the marker peaks n-tetracosane ($n\text{-}C_{24}H_{50}$) and n-octacosane ($n\text{-}C_{28}H_{58}$). The separation number (z) is given by the relation:

$$z = t_r/(b_{\frac{1}{2}}a + b_{\frac{1}{2}}b) - 1 \qquad \text{(eq. 2.3.)}$$

where t_r is the difference in retention between the marker substances and $b_{\frac{1}{2}}$ is the peak width at half height of the marker compounds, $n\text{-}C_{24}H_{50}$ (a) and $n\text{-}C_{28}H_{58}$ (b).

(b) Consideration of flow rate. We have seen how in theory the HETP is influenced by several diverse factors (see: van Deemter equation, p. 8). The achievement of maximum efficiences (minimum HETP) in practice, after the selection of column, temperature, and carrier gas, depends on the velocity of the carrier gas. While in some cases other experimental factors influence the selection of flow rates, these columns were generally operated at linear gas velocities, that is at flow rates, which maximize their efficiencies and thus their usefulness in the analysis of complex mixtures. The experimental consideration of the carrier gas linear velocity ($\bar{v}_1$) with respect to the minimization of HETP and the separation of closely-eluted substances was undertaken for these columns. The relationship of column flow rate to column length, temperature and

vent restriction in the dry injection system is treated in more detail later (Section 2.2.3.). A 50m Silanox-type glass (PLOT) OV-1 column was installed in a gas chromatograph and operated in the normal way.

Two test solutions were prepared. The first consisted of equal amounts of n-octadecane ($n\text{-}C_{18}H_{38}$: L. Light & Co., Colnbrook England) and n-octa dec-1-ene ($n\text{-}C_{18}H_{36}$: Analabs, Inc., Hamden, Conn., U.S.A., No.18114-472) in hexane solution (0.5mg/ml each). The second test solution consisted of 0.5mg each of 5α-androstan-3α-ol (Dr. G.F. Woods, Organon Research Laboratories, Newhouse, Lanarks.), 5α-androstan-3β-ol and 5-androsten-3β-ol (Medical Research Council Steroid Reference Collection) as the TMS ethers prepared in 50 per cent (v/v) bistrimethylsilylacetamide in dry pyridine, 60°C for 1 hour. Excess reagent and solvent were removed under a stream of dry nitrogen and taken up in hexane (0.5mg/ml per component). An aliquot of 1µl of each of the above solutions was applied to the probe of the dry injection device (Section 2.2.3.) and the resulting chromatograms were obtained at several vent settings previously calibrated for flow. HETP was calculated for 5α-androstan-3β-ol TMS according to Equation 2.2. and the separation between the two pairs of incompletely resolved peaks estimated by the per cent valley (%val) between them.

$$\%val = (\bar{h} - h_{min})/\bar{h} \times 100 \qquad \text{(eq. 2.4.)}$$

where $\bar{h}$ is the average height of the two peaks and h_{min} is the height of the minmum between the two partially resolved peaks. The results of these calculations appear in Table 2.2.

Table 2.2. Linear gas velocity and resolution and HETP

$\bar{v}_1$ (cm/sec)	flow rate (ml/min)	%val ($n\text{-}C_{18}$/ $n\text{-}C_{18}$-1-ene)	%val (5α-A*-3β-OTMS/ 5-A-3β-OTMS)	HETP x 10^{-3}m (5α-A-3 -OTMS)
121	14.2	22	51	4.6
65.8	7.6	43	62	3.0
25.3	3.0	73	89	1.6
14.6	1.7	76	89	1.4
7.3	0.85	67	91	0.95
4.4	0.51	54	87	1.6

* A = androstane

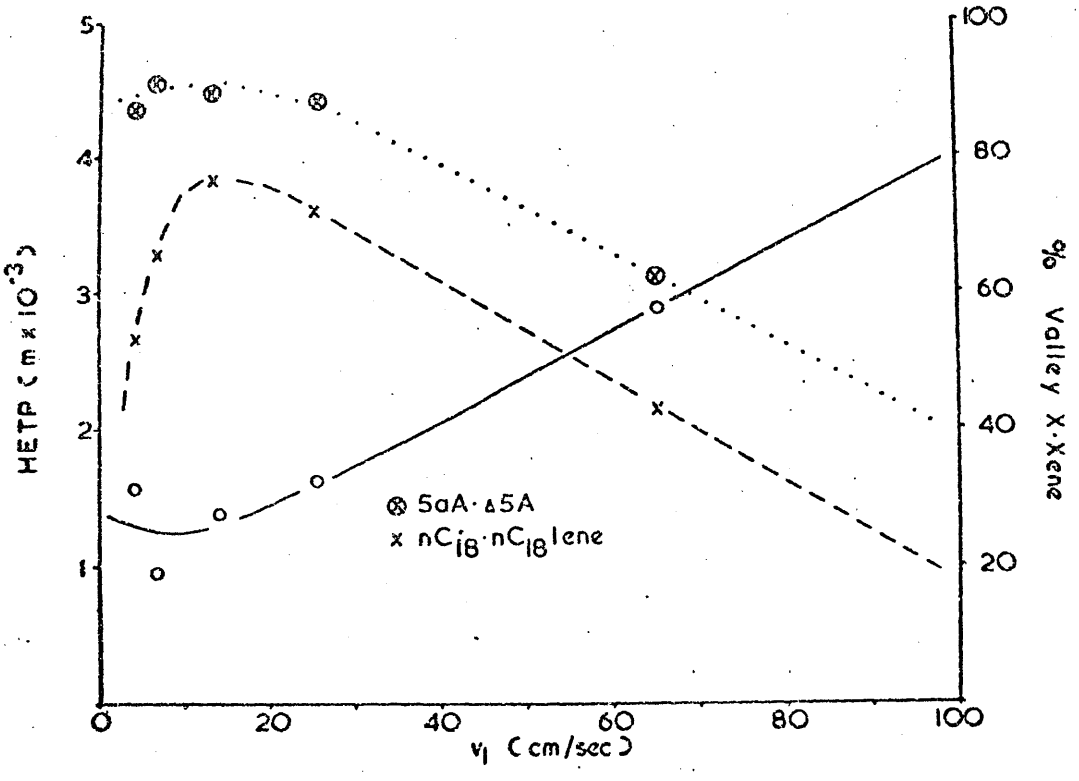

Figure 2.1. The influence of carrier gas velocity on HETP and efficiency of separation of closely-eluted substances. T = 230°C, carrier gas helium, column 50m Silanox-type (PLOT) OV-1 installed in PE F11 (FID) with dry injector.

The lower curve (solid line) represents the hyperbolic relation between HETP and average linear gas velocity, $\overline{v_1}$, for 0.5µg 5α-androstan-3β-ol TMS ether.

The upper two curves represent the per cent valley, measured as the ratio of the depth of the valley between two partially resolved peaks and the average height of the two peaks x 100. The broken curve in the separation of 0.5µg each of n-octadecane (designated nC_{18} for brevity) and n-octadec-1-ene (designated nC_{18}lene for brevity) and the dotted curve that of 0.5µg each of 5α-androstan-3β-ol TMS ether (designated 5αA for brevity) and 5-androsten-3β-ol TMS ether (designated 5A for brevity).

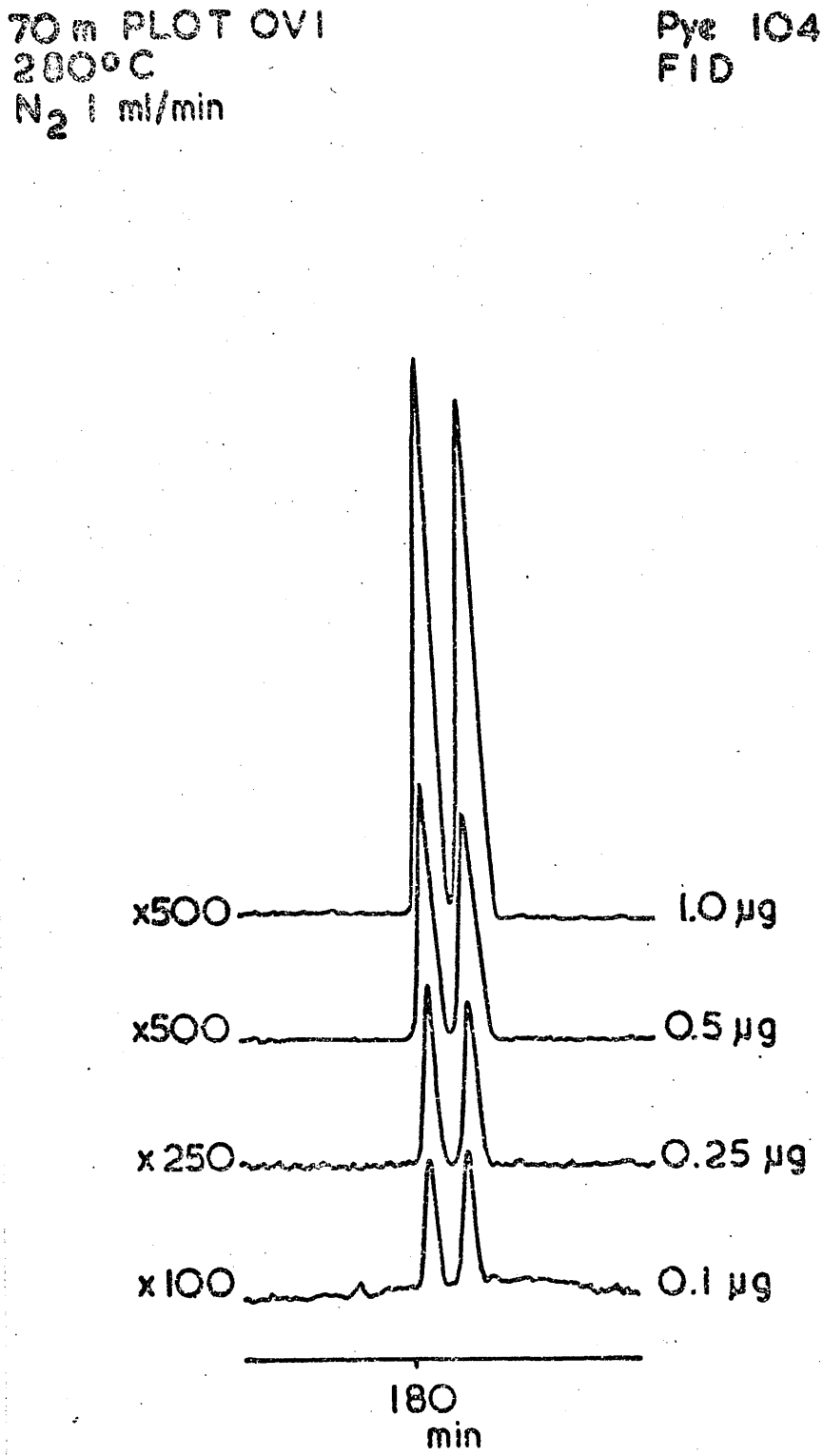

Figure 2.2. Effect of increased loading on separation of 5α-cholestan-3β-ol TMS ether (a) and cholesterol TMS ether (b). 70m Silanox-type (PLOT) OV-1 column at 280°C with nitrogen carrier gas at 1ml/min.

facing page 48.

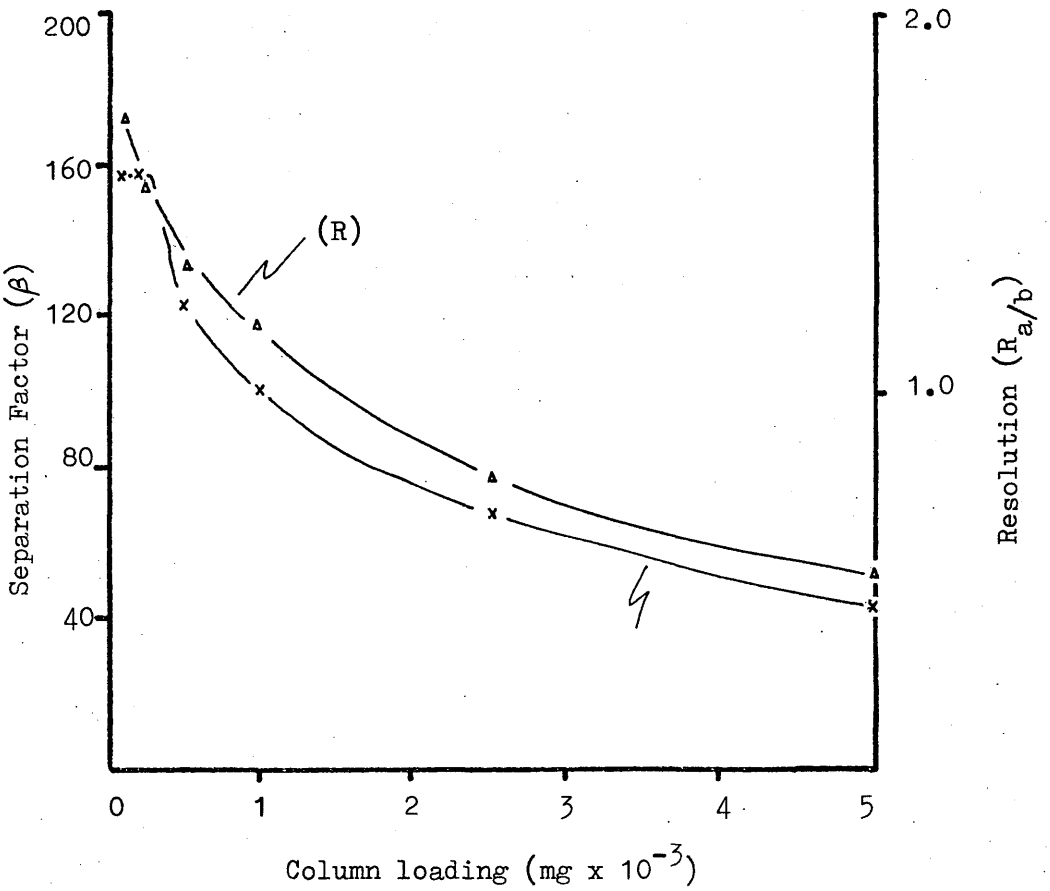

Figure 2.3. Column performance with increasing column loading. 70m Silanox-type (PLOT) OV-1 at 280°C with N_2 carrier gas, 1ml/min.

Right ordinate: Resolution (R) of 5α-cholestan-3β-ol TMS ether and cholesterol TMS ether.

Left ordinate: Separation factor (β) for 5α-cholestan-3β-ol TMS ether.

Figure 2.1. represents the above data graphically. The optimum carrier gas linear velocity as indicated by minimum HETP appears to be 10cm/sec, corresponding to a flow rate of 2ml/min. The flatness of the curve for the %val for the model separation of 5α-Δ^5 sterols indicates that the penalty paid for higher flow rates and the correspondingly shorter analysis times is minimal to a point of diminishing returns near 25cm/sec, a flow rate of 5ml/min.

(c) Consideration of sample loading. If the quantity of a substance introduced into a column exceeds the capacity of a theoretical plate, then the column performance diminishes. Load capacity is then conveniently understood as the quantity of pure substance which reduces the column performance to 90 per cent of the maximum value; or alternatively, reduces the separation factor (β) by 5 per cent.[194]

$$\beta = t_{dr}/b_{\frac{1}{2}} \qquad \text{(eq. 2.5.)}$$

where t_{dr} is the uncorrected retention time of the test substance and $b_{\frac{1}{2}}$ is the peak width of the substance at half height. This separation factor is a direct index of column efficiency as it is expressed in peak sharpness. Figure 2.2. and Figure 2.3. demonstrate the effect of load capacity on the separation of the 5α-Δ^5 sterol pair, 5α-cholestan-3β-ol TMS ether and cholesterol TMS ether. This structural pair is of general significance in the analysis of naturally occuring steroid and sterol mixtures and was adopted as a model system. A test mixture of these two sterols as TMS ethers was prepared according to the standard procedure. Standard solutions were prepared by careful dilution and injected on a 70m Silanox type glass (PLOT) OV-1 column installed in a Pye Model 104 gas chromatograph at 280°C ,with nitrogen as carrier gas at 1ml/min.

By convention, resolution (R) may be expressed as the ratio of the distance between two peak maxima (Δt) and the average base width of the two peaks. When the peaks have the same width (i.e. equal quantities of similar materials are eluted very near one another) taking the base width of the second peak (w_{b2}) for the average value.[195]

$$R = \Delta t/(w_{b1} + w_{b2})/2 = \Delta t/w_{b2} \qquad \text{(eq. 2.6.)}$$

facing page 49.

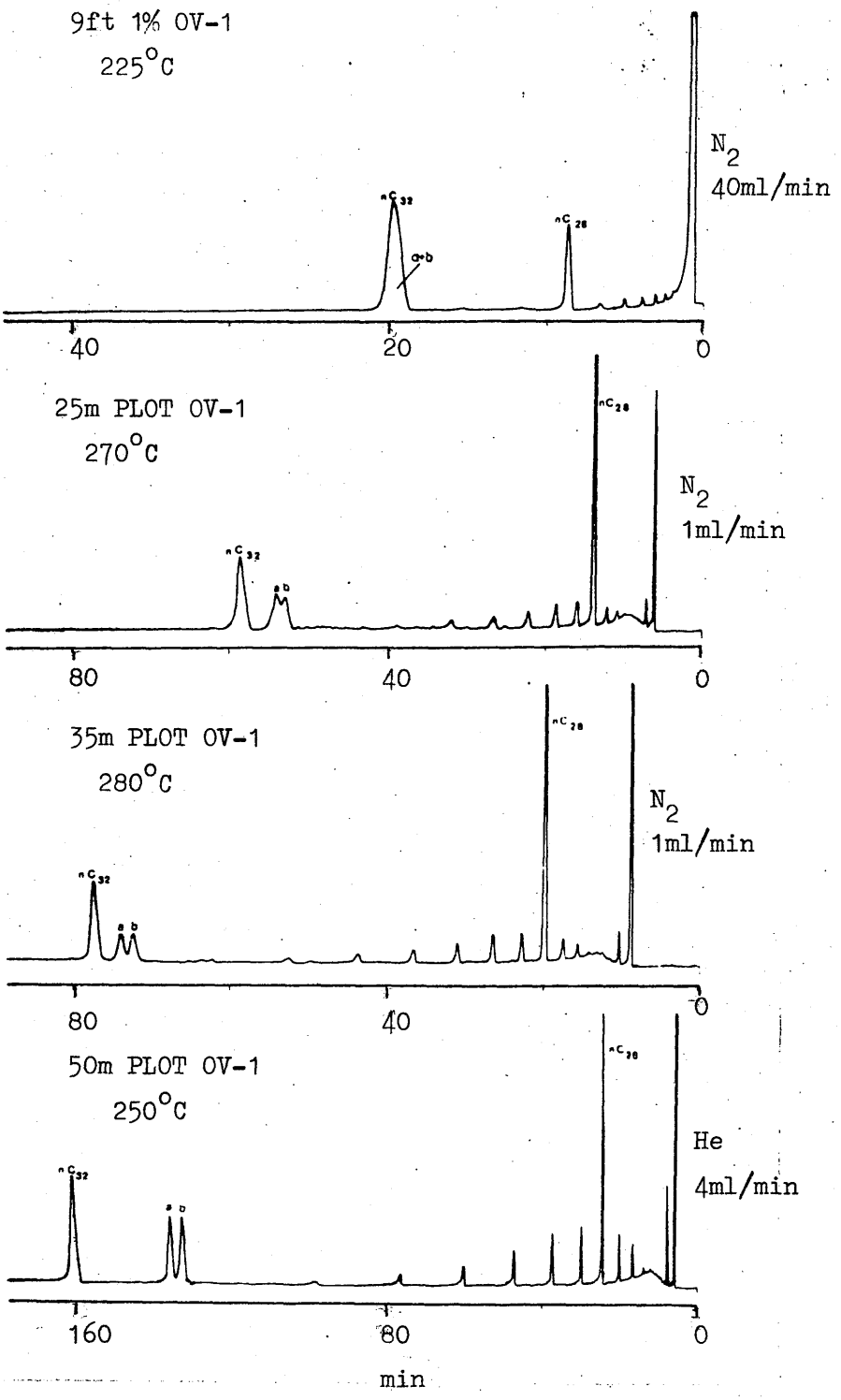

Figure 2.4. Comparison of the separation of 5α-cholestan-3β-ol TMS ether (a) and cholesterol TMS ether (b) on packed OV-1 and three Silanox-type (PLOT) OV-1 columns installed in a Pye 104 (FID) gas chromatograph.

In the case of the resolution of R = 1 the separation is 85 per cent complete and at R = 1.5 the separation is 99.7 per cent complete, a "base line" separation. In figure 2.3. shows the effect of column loading on the separation factor (β) of 5α-cholestan-3β-ol TMS ether and the resolution (R) of 5α-cholestan-3β-ol TMS ether and cholesterol TMS ether. At loading above 0.25μg the separation factor (β) drops abruptly from the maximum of 160, corresponding to a column efficiency measured for 5α-cholestan-3β-ol TMS ether of 87,500 theoretical plates. The resolution (R) decreases more smoothly over the range of sample loading. The effects of increased loading are more obvious in Figure 2.2. The peak pair shows decrease in resolution (R) and the characteristic retarded onset and rapid decay of overloaded peaks. None-the-less, the usefulness of the separation remains. A practical working limit for sample loading would be in the vicinity of 1μg per component.

2.1.4. Conclusion. Figure 2.4. summarizes the progress in column performance above with respect to that obtainable with conventional packed columns. In this work the performance factors obtained are modest in comparison to those quoted by German and Horning (n = 1400-1600 theoretical plates/m). This may be partially the result of the somewhat larger internal diameter of these columns (0.5mm) in comparison to those of other workers (0.25 - 0.3mm). However, the diameter increase offers higher phase loading and higher sample capacity important for g.c.-m.s. applications. From the literature it also seems possible that elaborations involving surface treatment[170] or static coating procedures[126] might be advantageous. However, it was determined to invest this effort in exploration of the application of the columns in hand. Columns of this type have increased the efficiency available for g.c. and g.c.-m.s. by an order of magnitude.

2.2. The Gas Chromatographic System

2.2.1. Introduction. The development of open tubular columns followed the early work on the theory of gas liquid chromatography on packed columns.[196] While the importance of these advancements was generally acclaimed, commercially available chromatographs continued to be designed primarily with packed columns in mind. The use of chromatographic equipment unsuitable for the task has

retarded the growth in applications for open tubular columns.[23] The elements of the chromatographic system, injector, connectors, and detectors are not necessarily interchangeable or easily adaptable between the two systems. The differences in column dimensions and in the volumetric gas flow are the obvious points upon which the design considerations of the two systems diverge. The volumetric flow in packed columns (30-60ml/min) is substantially higher than that for open tubular columns (1-5ml/min). However, with the substantially smaller cross sectional area in open tubular columns the linear velocity of the carrier gas is higher. Large volumes in injectors, connectors and detectors employed for packed columns must be reduced to maintain these high linear velocities when using open tubular columns. If a large capacity appears in series with an open tubular column, the resulting reduction in linear velocity increases the opportunity for diffusion and mixing of the sample. The effect is to reduce or eliminate the efficiency of the separation obtained. Adaptation of existing chromatographs or the construction of purpose built instruments must be carried out with these fundamental design considerations in mind.[197] Where glass columns are to be used, consideration must be given to the fragility of the glass spirals and to connectors which must be inert, temperature stable and leak free. Additional problems are posed by the analysis of relatively involatile and thermally labile materials. With the higher operating temperatures all elements of the system must be free from active sites which would result in absorption. With the recent enthusiasm for glass open tubular g.c. these problems have recently been re-discussed in the literature.[198,199]

2.2.2. The injector. It is a basic criterion for any gas chromatographic sampling system that the sample be introduced and vaporized instantaneously and completely. The sample plug must occupy a small volume and band spreading by back diffusion must be avoided. In open tubular chromatography this is accomplished by minimizing the effective volume of the injector and maintaining high linear velocity of the carrier gas in the sampling volume. The techniques employed fall into two categories, viz. solventless "dry" injection, and injection of solutes in solutions of volatile solvents or as liquids.

(a) Liquid injection. The injection of substances for analysis by gas chromatography as solutions in a volatile solvent has been the method of choice for most packed column applications.

The direct application of a solution to the top of the heated column or in a heated zone directly above the column fulfils all the requirements for sample introduction. In addition, handling of solutions allows for the convenient measurement and quantitative transfer of amounts of material which would otherwise be unmanageable.

The smaller capacity of open tubular columns and the lack of good micro-sampling devices initially led investigators to devise indirect sampling techniques for liquids and solutions.[197,200] The solute in solution is injected into a heated volume where solute and solvent are vaporized and swept by carrier gas to a tee device which delivers a portion of the vapour stream to the column and the bulk to a pneumatic restrictor and subsequently to vent. The amount of solute vapour delivered to the column is determined by the ratio of the flows through the column and that vented through the pneumatic restrictor ; i.e. the split ratio of the device. This ratio is adjustable by varying the restriction. This feature provides two degrees of control of the amount of material applied to the column, the amount and concentration of solution injected and the ratio of the splitting device. Initially these devices suffered from a molecular weight bias, the more volatile materials being favoured for venting. The heated vaporization and mixing chamber also provided opportunity for absorption and decomposition of sensitive materials.

Problems of sample introduction were substantially simplified by the introduction of precision microsyringes, and the injection device of Ettre[201] went a great way in providing "linear" splitting for all but the most extreme situations. Glass lining of the vaporization volume[201,202] and all-glass splitting devices[203] reduced or eliminated catalytic activity, but the necessity for maintaining these devices at relatively high temperatures remained a problem for the chromatography of involatile and thermolabile materials of biological origin. A splitter injection system incorporating a short precolumn and splitting of sample vapour after passage through this column has appeared.[204] This offers the best prospect for stability and inactivity for the analysis of steroid profile mixtures. The precolumn protects the analytical column and eliminates the possibility of injecting aerosols of non-vaporized and involatile material onto the column. This is particularly important in the analysis of materials from biological extracts. The discrimination of splitters on the basis of molecular

weight, the temperatures required to achieve complete and rapid evaporation of high boiling compounds, the uneconomical use of valuable materials lost by venting and the complexity of the apparatus militate against stream splitting. Direct injection offers an alternative. However, the effect of solvent remains a subject of some controversy.

The "solvent shock" has been considered important to avoid. It has been blamed for rapid column deterioration, for obscuring early peaks and for adversely affecting retention data by producing transient changes in the stationary phase. Measures against the adverse effects of solvent have included the injection of solutes in a solution of involatile silicone oil[205] and a number of cold zone procedures. All are more or less elaborate. One involves the use of a cold trap at the injector which collects solute and allows passage of solvent.[206] After the solvent has passed the heated column, the cold trap is abruptly heated and the solute swept by carrier gas onto the column. The solvent may be prevented from traversing the column at all by applying the sample in solution to a precolumn[207] or a separate section of capillary[208,209] which after the solvent has been removed by a gas stream is connected to the chromatography column and heated to volatilize the solute and allow it to pass onto the column. The simplest and most useful technique for this splitless injection involves the injection of the solute in volatile solvent onto the open tubular column at a temperature substantially below that of analysis. After the solvent has passed the cool column, the temperature is abruptly increased to an initial point and the analysis carried through by temperature programming. This technique has been applied to good effect to fatty acid methyl esters,[161] cigarette smoke constituents[161,203] and steroids.[209-212]

Direct injection with no measures for removal of solvent has been employed. A gold-lined, low internal volume direct-injection device has been described.[213] Grob and Grob have recently presented convincing evidence for the desirability of the solvent for maximization of chromatographic efficiency of open tubular columns.[214] The solvent serves to suppress the migration of sample components at the point of injection, thus concentrating the vapour plug.

(b) Dry injection. Vaporization of solute in the injector in absence of solvent circumvents the problem mentioned above.

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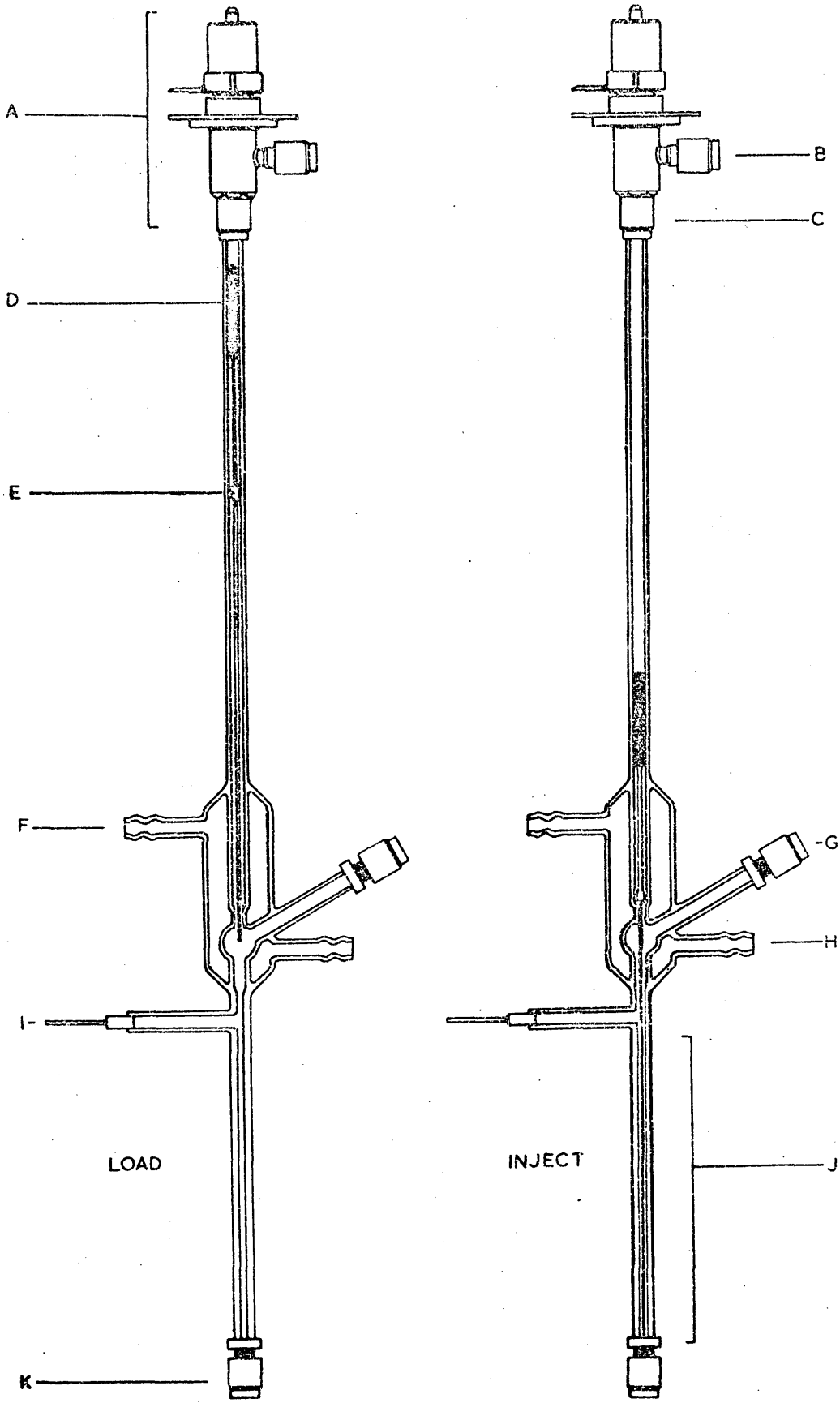

Figure 2.5. Dry injector for open tubular gas chromatography with variable restrictor (A) and water cooling.

Syringes adapted for the injection of solids through a conventional septum injection port offer some advantages.[215,216,217] Various dry injection techniques evolved from the method of Menini and Norymberski[218] have been applied with success to packed and open tubular columns. In this technique, samples in volatile solvent are applied to spirals, rods, tubes or gauzes of platinum, silver, glass or stainless steel outside the chromatograph. The solvent is allowed to evaporate and the remaining sample thus fixed to the support which may either be manually or automatically dropped into the heated evaporation zone above the column. The disadvantage of the system is the large volume of the evaporation zone required to accommodate several sample holders for repeated injections. The technique is the basis of two commercially available dry injection systems[219,220] and has been successfully employed for steroid profiles on open tubular columns.[131,221]

2.2.3. Glass dry injection device. Figure 2.5. shows the glass dry injection device constructed for use in this work. This device is a one-piece version of the dry injector constructed by van den Berg and Cox.[222] This in turn is derived from the dry injector described by Ros[223] and is similar to an earlier concept.[224] Figure 2.5. with key opposite shows the injector in position for loading and for injection. The carrier gas enters the injector (I) and is split: a portion of the stream (15-25ml/min) passes the valve restrictor and is vented to atmosphere, the remainder (1-10ml/min) passes down to the column connector (K) and to the column. The steel stub (D) allows manipulation of the probe by a magnet from loading to inject position.

Sample loading is accomplished through a silicone rubber septum (G), with the probe in the raised position, by means of a microlitre syringe. The sample in a volatile solvent (hexane or ethyl acetate) is applied to the tip of the probe, a glass knob approximately 1mm diameter. Volumes up to 5µl can be conveniently transferred in a single operation or the procedure repeated where larger volumes are required or two solutions are to be co-injected.

Key to Figure 2.5.

A. variable restrictor valve (Edwards High Vacuum Ltd., Crawley, Sussex); B. solvent vent; C. rubber "O" ring union; D. steel stud; E. glass sample probe; F. cooling water inlet; G. septum inlet; H. cooling water inlet; I. carrier gas inlet; J. injector heater zone; K. Glaslok^{R} union to column connector.

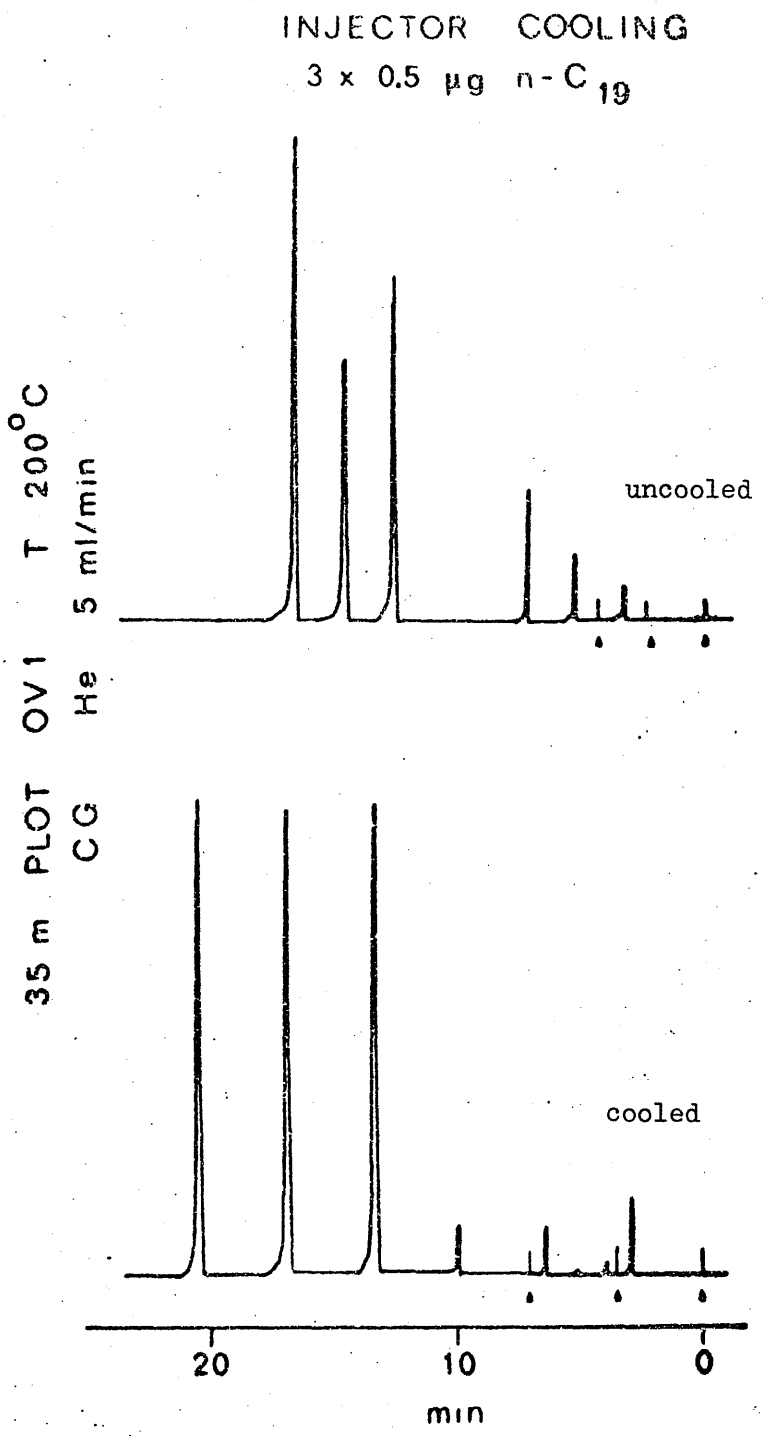

Figure 2.6. A comparison of the total ionisation current (TIC) chromatograms of three successive injections of 0.5µg nonadecane ($n\text{-}C_{19}H_{40}$) from the dry injector, cooled and uncooled, on an open tubular column installed with gas make-up adaptor in the LKB 9000. Injector heater temperature 220°C.

the solvent is evaporated by the stream of gas flowing toward the vent. After an interval the probe is then lowered and the tip with solvent free sample passes into the vapour swept through the column connector to the column itself. The carrier gas passes easily through the annular space between the probe and the wall of the injector. There is no measurable change in carrier gas flows between the loading and inject positions.

In the inject position the needle fills the space above the column connector and eliminates dead volume in the injector. The linear velocity of carrier gas is high in the injector and opportunities for diffusion are minimized. Peak broadening due to slow vaporization is minimized by the heating of the area of the injector where the tip of the probe comes to rest. This also serves to make injector performance independent of column temperature. In isothermal operation the injector is maintained 20°C above column temperature, and for programming the temperature is selected to ensure rapid vaporization of all components of the sample onto the cooler column.

2.2.4. Injector performance. This type of dry injector has been evaluated by Luyten[225] and employed with open tubular columns for the analysis of steroids by a number of workers.[129,226,227,228] Evidence indicates that there is less than 1 per cent loss in column resolving power associated with the injection device and that the quantitative performance compares favourably with that obtained by direct liquid injection. This is confirmed by our own experience.

The amount of heat leakage to the injector from injector and column heaters varied with instrument installation. It was found particularly significant on the LKB 9000 whose massive injection heater required the cooling of the injector even for the most qualitative of applications. Figure 2.6. shows the improvement in injector reproducibility and yield produced by cooling with circulating water at 19°C. Successive injections were performed of 0.5µg of n-nonadecane ($n\text{-}C_{19}H_{40}$: designated $n\text{-}C_{19}$ for brevity) on 35m Silanox-type glass (PLOT) OV-1 column installed in the LKB 9000 at 200°C with the injector heater at 220°C. A 10µl syringe was employed in transferring a solution of 0.5mg n-nonadecane (L. Light & Co. Ltd., Colnbrook, England) in 1ml Nanograde hexane. The upper trace shows the result for the

uncooled injector. The variation in injector yield is a consequence of distillation of the hydrocarbon prior to the injection, the interval between injections being 1.5min. The lower shows the improvement of reproducibility and yield with cooling. The interval between injections has been lengthened to 3 minutes but the reproducibility is that that might be expected from the transfer operation with microsyringe.

The use of the mass spectrometer to improve sensitivity and detectability in g.c.-m.s. by monitoring single m/e values is well understood.[71,82,229] The characteristic increase in mass flow rates in open tubular columns makes their combination with 'selected ion monitoring' (SIM) of particular interest. This dry injection system has been employed with open tubular columns interfaced with mass spectrometers directly[228] and through jet separators[226,227] to obtain data on small amounts of steroid materials by both focused ion and full scan mass spectrometry. Luyten states that "structure dependent" losses occurred at sub-nanogram levels, an observation which he attributes to absorption and thermal degradation in the metal molecular separator. Whether direct coupling of the columns to the source of the mass spectrometer would eliminate this affect remains unclear. It is undeniable that opportunity for absorption exists at all points in the chromatographic system: thus yields of the injector, and sensitivity of the combined apparatus. The yield of the injector at low sample levels was investigated.

A 50m Silanox-type glass (PLOT) OV-1 column was installed in the LKB 9000 with dry injection system in the normal way. The glass lined metal tubing (GLT)* used as column inlet connector was replaced by a silanized section of narrow glass tubing, the column connection being of heat shrinkable PTFE tubing (Phase Separations Ltd., Queensferry, Flints.). This was to eliminate the possible site for absorption in the metal end of the GLT. The LKB 9000 was adjusted to monitor at m/e 372 according to the previously published procedure,[71] the molecular ion of 5α-cholestane. Successive aliquots of a carefully diluted solution of 5α-cholestane (Steraloids Ltd., Croydon, England, No.C720) were injected in amounts from 5 to 40ng. The injections were repeated with coinjection of 2μg of 5α-androstane. The analogue signal for m/e 372 was monitored. The

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* Scientific Glass Engineering Pty. Ltd., 657 North Circular Road, London, NW2 7AY.

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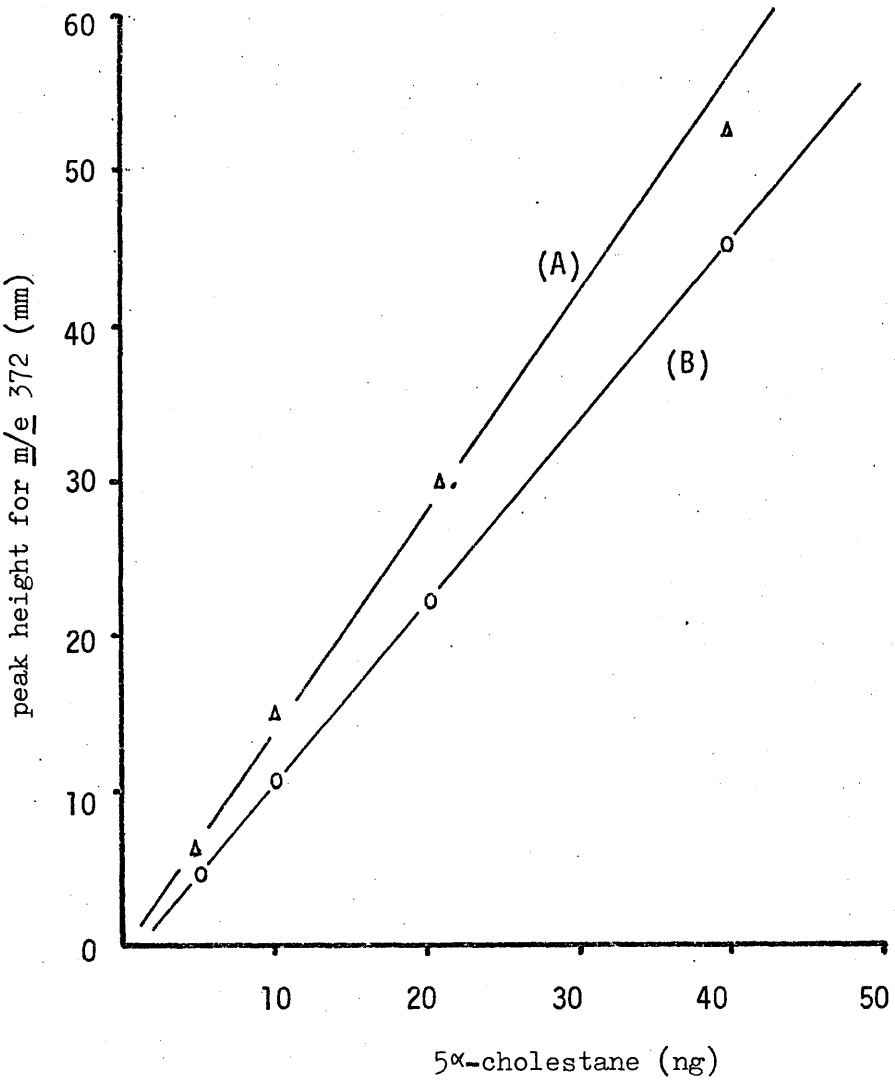


Figure 2.7. Single ion monitor intensity m/e 372 versus quantity of 5α-cholestane injected by cooled dry injection device with (A) and without (B) 2µg of 5α-androstane co-injected as carrier. 50m Silanox-type glass (PLOT) OV-1 column at 260°C, injector heater at 280°C, helium carrier gas at 5ml/min, multiplier sensitivity 250 (3.1kV) on 100 mV full scale potentiometric recorder.

results are shown in Figure 2.7. The enhancement of the signal for 5α-cholestane with co-injection of a large amount of more volatile material seems to indicate some evaporation of material from the probe. This evaporation, measures for cooling having been taken, is negligible for larger amounts of material and significantly reduced for small amounts by the presence of a large excess of more volatile material. However, the excess may also be functioning as a carrier, suppressing absorption at other points in the system.

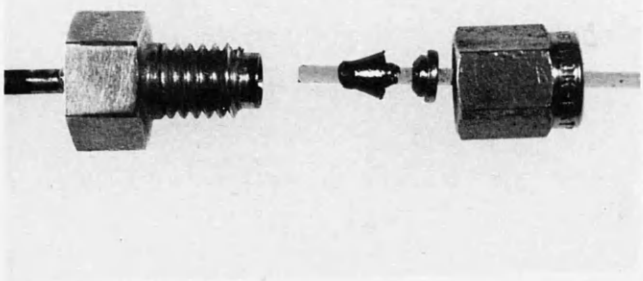
2.2.5. Column flow control. Carrier gas flows in packed columns are controlled by a variety of commercially available flow controllers and mass flow regulators. These function well for isothermal and temperature programming so long as the flow remains above 10ml/min. The lower volumetric flows required by open tubular columns are controllable only by controlling inlet pressure. A constant pressure drop along the column produces a constant flow of constant average linear velocity of carrier gas. This is termed isobaric operation. Under isobaric conditions the flow of carrier gas falls in a more or less linear fashion with a rise in temperature.[230] If the temperature changes due to column oven instability, or in temperature programmed operation, the flow will alter substantially. This has consequences in the behaviour of detection systems which are sensitive to flow variations. Flame ionization detectors are substantially unaffected. In respect of retention data obtained by open tubular gas chromatography, these may be more sensitive to oven temperature variation with isobaric flow control. In relation to carrier gas flow control, the dry injection system is useful. The metering valve serves as a variable leak for the stream of gas, which removes solvent from the sample but also controls the inlet pressure of the column and thus column flow rate. Flow rates quoted through this work are, unless other calibration is specified, as volumetric flow for a specific column and injector restrictor setting obtained by a 1ml bubble flow meter at room temperature, uncorrected for the compressibility of the gas, the vapour pressure of water or the eventual temperature at which the analysis will be carried out.

2.2.6. Column Connectors. The elimination of excess volume and active sites are also significant for connecting tubing and connectors.

facing page 57.

PLATE 1

a.



Glass-graphite GLT connector, disassembled after use. Graphite ferrule formed to outside diameter of column.

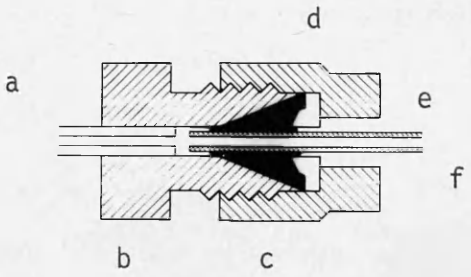
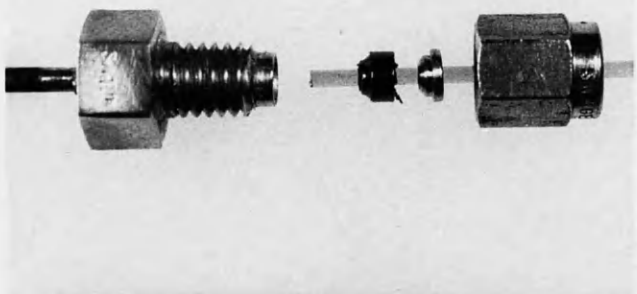


Diagram of assembled glass-graphite GLT connector. (a) glass lined metal tubing (GLT), (b) Swagelok union soldered in place, (c) graphite ferrule, (d) back ferrule, (e) Swagelok captive nut, (f) open tubular glass column.

b.



Glass-graphite GLT connector prior to assembly.

In tubing and connectors a larger diameter than the column will result in a corresponding reduction in linear velocity and the enhanced opportunity for peak broadening due to diffusion. In addition, geometry which disrupts the laminar flow of carrier gas may cause peak broadening and "tailing". The connecting tubing in the chromatographic systems described here is commercially available glass lined metal tubing (GLT)*. The tubing is of 1/16" outside diameter and the glass liner 0.5mm internal diameter. This tubing may be connected to equipment by standard Swagelok^{R} fittings or Pye 4 B.A. tube connectors or silver soldered in the conventional way. This tubing provides an inert, mechanically sound connecting tube which can be shaped to accommodate the capillary spiral. This has been found to minimise time-consuming manipulations and breakages.

The problem of the union between connecting tubes, detectors injectors and glass open tubular columns has been approached in a number of ways. Silicone rubber 'O'-rings and septa[152,231] have been used, also plastic ferrules and epoxy cement unions.[192] The most popular connector is heat shrinkable PTFE tubing because of its convenience and inertness.[225-228] These systems all suffer to a greater or lesser degree from thermal instability. A particular problem in the coupling of open tubular columns directly to a mass spectrometer has been encountered with heat shrinkable PTFE tubing which becomes air-permeable above 170^{o}C.[232]

For this work a thermally stable, inert and leak-free connector has been obtained using graphite front ferrules** with Swagelok^{R} unions soldered to GLT. Plate 1 shows the arrangement of this union. The GLT is silver soldered to a 1/16" Swagelok^{R} union drilled out to a diameter of the tubing. The graphite front ferrule, metal back ferrule in the normal position, and captive nut are arranged (Plate 1b), the column end inserted into the union and the union closed and tightened. In this glass-graphite-GLT union the column closely approaches the lining of the GLT. The graphite ferrule is compressed to the diameter of the column and into a thermostable, leak tight seal which is unique to the column

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* Scientific Glass Engineering Pty. Ltd., 657 North Circular Road, London, NW2 7AY, Glass Lined Metal Tubing (GLT).

**Scientific Glass Engineering Pty. Ltd., Graphite front ferrules Cat. No. GFF/16

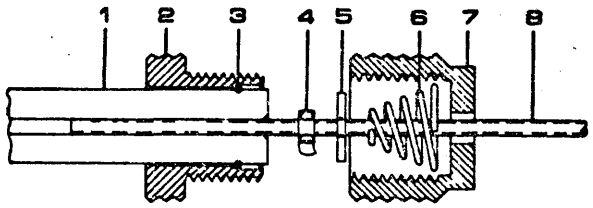

Figure 2.8. Dry Injector Glaslok^{R} connector.* Precision bore glass tubing at base of injector (1), threaded sleeve (2), retaining ring (3), PTFE gas-tight seal (4), compression washer (5), compression spring (6), captive nut (7), connecting tubing to column inlet connector, glass or GLT (8).

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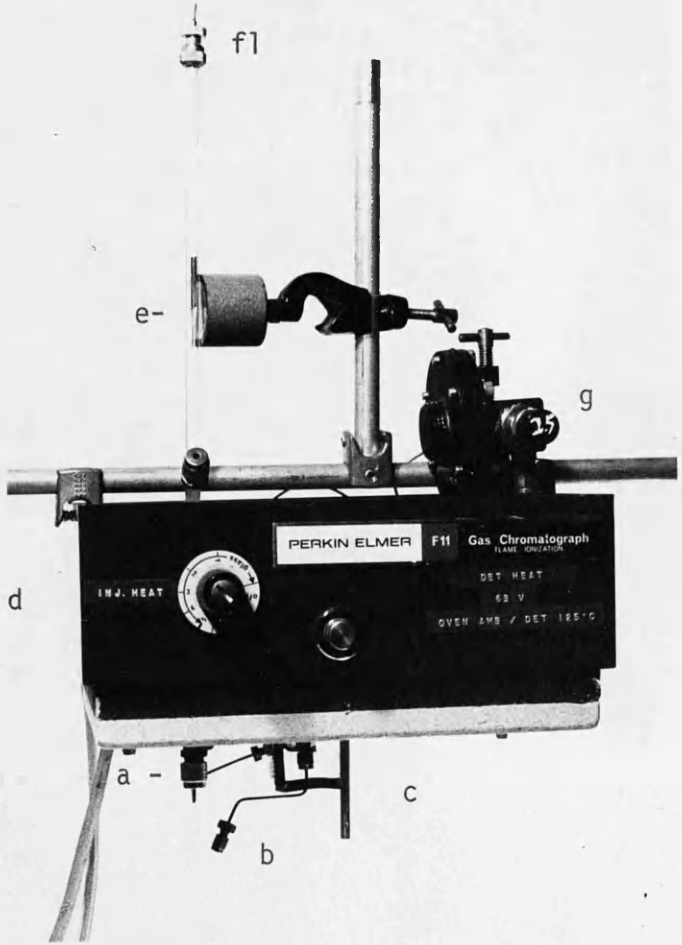

Plate 2 Perkin-Elmer F11 analyser unit modified for use with open tubular glass columns. See key opposite.

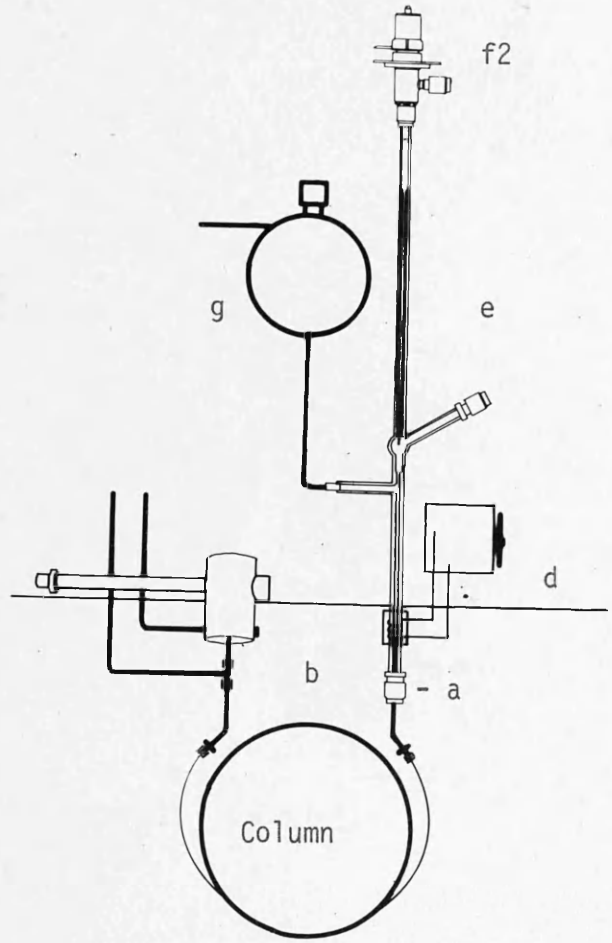

Figure 2.9 Schematic diagram of Perkin-Elmer F11 analyser unit modified for use with open tubular glass columns. See key opposite.

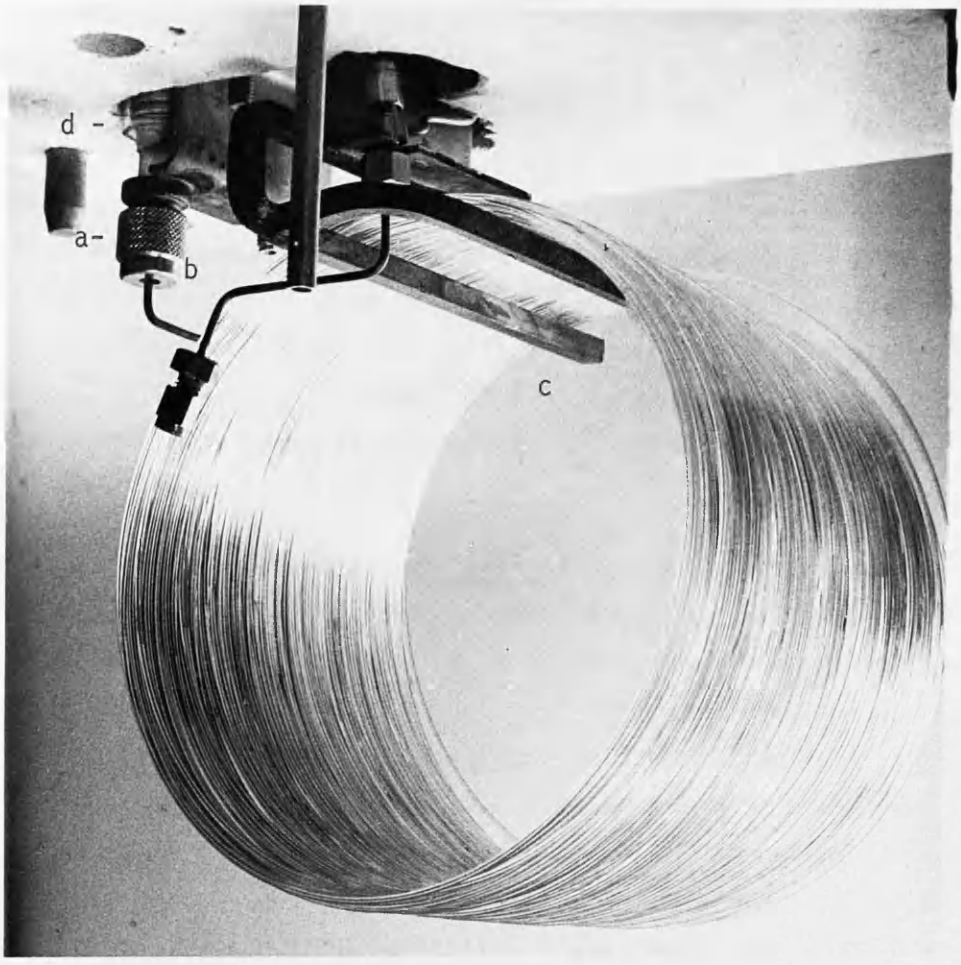

Plate 3 Column connector and support assembly of Perkin-Elmer F11 modified for use with open tubular glass columns. (a) Glaslok connector at bottom of injector, (b) Glass-graphite GLT connectors at column inlet and outlet, (c) column support fork, (d) 20w Pye injection heater and proportional controller, (e) dry injector, less cooling jacket, (f1) set vent restrictor, (f2) metering valve variable vent restrictor, (g) Brooks flow controller for carrier gas.

and re-usable (Plate 1a).

Figure 2.8. shows the Glaslok^{R} union used in connecting the injector to the chromatographic system. This method was convenient for making connections to GLT connecting tubing, all glass column connecting tubing employing heat shrink PTFE for the column union, or to a straightened portion of the column itself. The sort of connection chosen at the injector depends on whether inactivity is more important than strong, convenient connectors. In the use of the dry injector and glass or glass lined connecting tubing all glass surfaces in the chromatographic system were periodically cleaned and silanized with 5% (v/v) dimethyldichlorosilane (DMCS: Pierce Chemical Co., Rockford, Ill.) in toluene overnight at room temperature. The pieces were washed in AnalaR methanol, dried and re-installed.

2.2.7. Gas chromatograph arrangements. Two gas chromatographic instruments were used for open tubular chromatography in this work. Two different approaches were taken for the arrangement of injector, connecting tubing and columns. In the case of a Perkin-Elmer Model F11 equipped with flame ionization detector* an entire analyser unit was modified for open tubular columns, creating an instrument (Figure 2.9., Plates 2 and 3) wholly dedicated to this technique. In brief, the PE F11 (FID) system consisted in a Brooks flow controller**, (g) delivering carrier gas to the dry injector (e) mounted in the Marinite block of the analyser unit. The injector heater (d) is a 20W Pye heater unit (Pye Unicam Ltd., Cambridge, England, No.717190) and proportional energy control unit (Pye Unicam Ltd., Cambridge, England, No.718539) mounted on the unit. The Glaslok^{R} union (a) is soldered to the bracket beneath the unit and provides support for the injector. The GLT connecting tubes for inlet and outlet (b) are situated tangent to the column which is supported by two bars (c) mounted on the underside of the analyser unit. The column connecting tube is mounted on the flame ionization detector by a Swagelok^{R} union. The detector is unmodified and the only addition to the column oven

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* Perkin-Elmer Limited, Beaconsfield, Bucks., England, Model F11 Modular Gas Chromatograph.

**Brooks Instrument Ltd., Stockport, Cheshire, England, Model 8944.

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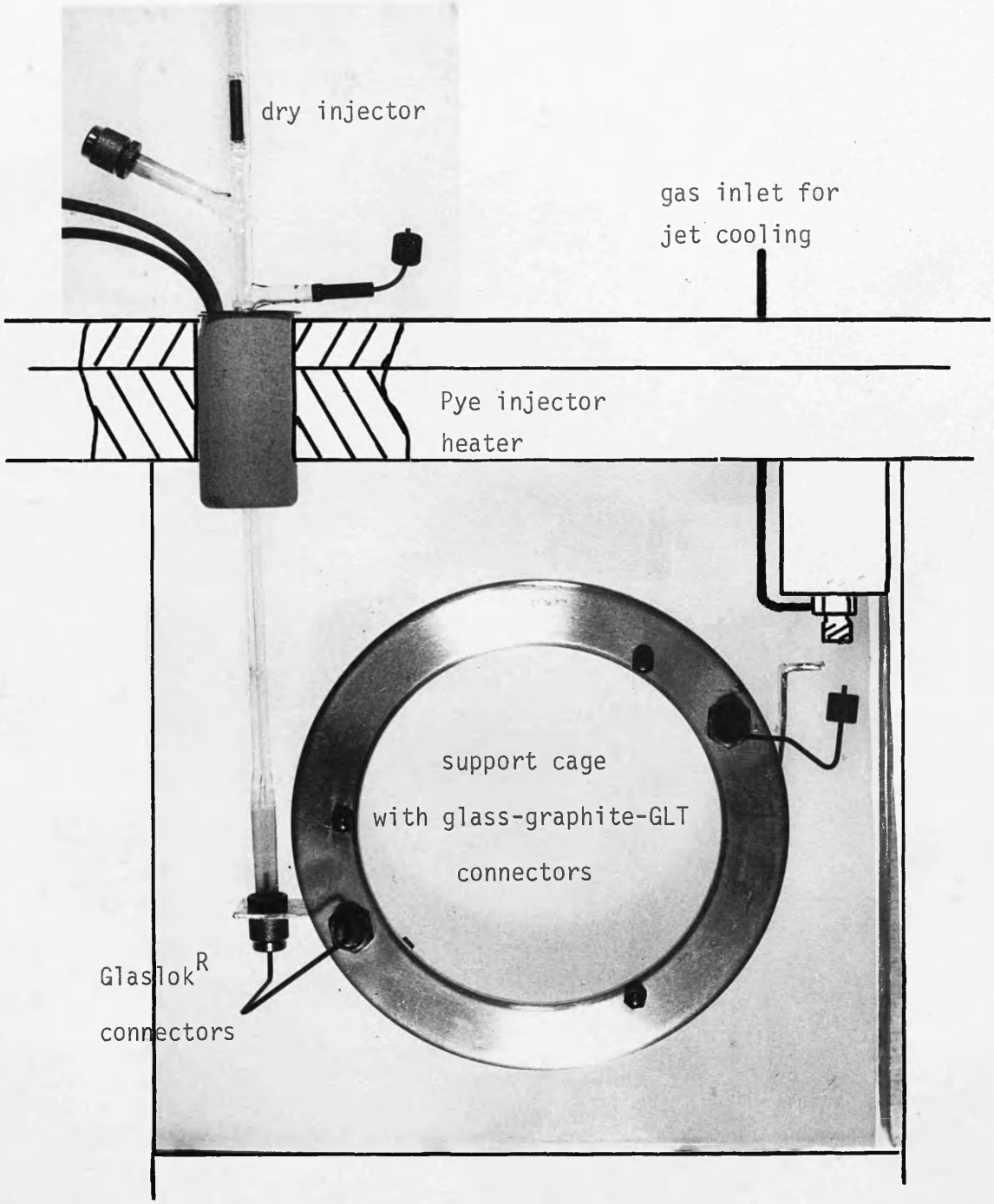

Plate 4 Arrangement of injector and column support basket for the use of open tubular glass columns in Pye Model 104 gas chromatograph.

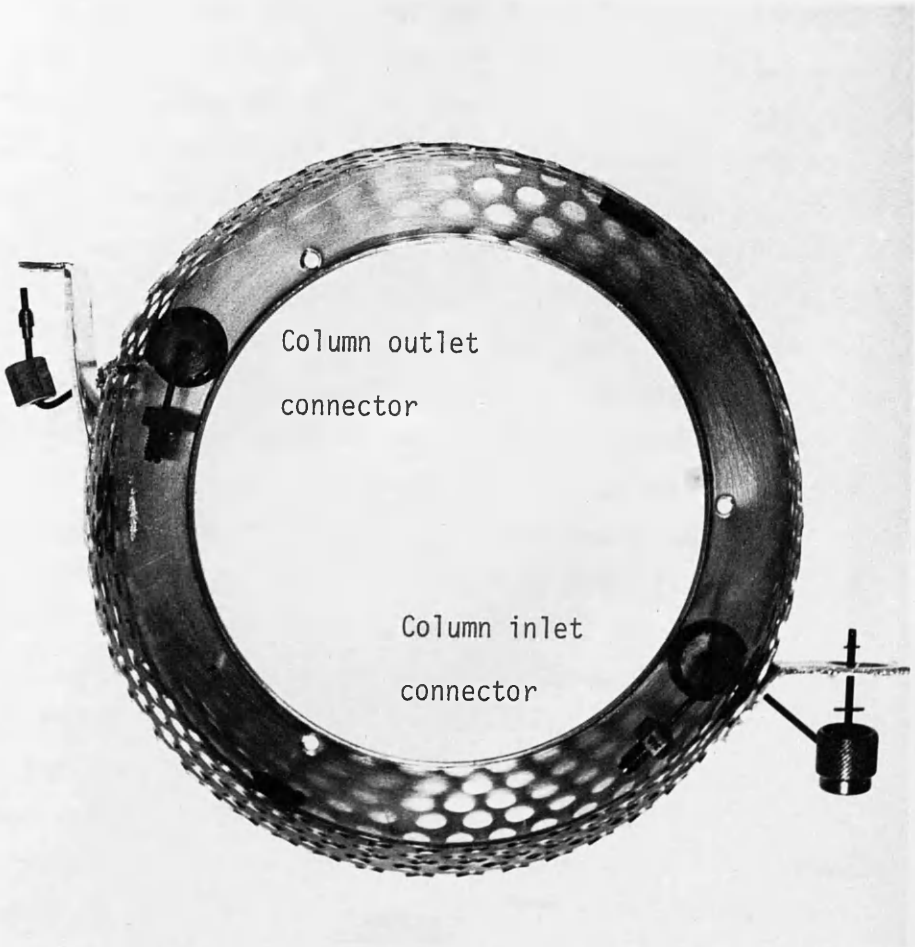

Plate 5 Interior of column support basket. Glass-graphite-GLT connector assemblies are rigidly mounted to the side of the support basket. The column is installed and a cover plate bolted in place to provide permanent support and protection in column and connectors.

unit is a 1.5" asbestos spacer at the upper edge to increase the depth of the oven by that amount.

The only modification of the Pye Unicam Series 104 gas chromatograph* is the provision for an ancillary flow of gas to be added to the column effluent at the detector inlet connector (Plate 4). This gas does not function as "scavenger" but serves to increase mass flow through the detector. This prevents the polarized jet of the detector overheating in the low flows encountered with open tubular columns by the addition of 25ml/min of carrier gas to column effluent.

The interchangable system devised for use with Pye 104 (FID) is facilitated by a commercially available support cage for open tubular columns**. This consists of a shallow aluminium basket equipped with appropriate portions of GLT and glass-graphite-GLT connectors. The column outlet connecting tube attaches to the detector inlet connector by means of a Pye 4 B.A. union. The dry injector is extended with a 10cm length of 0.5mm i.d. glass capillary. The support cage is supported by the Glaslok^{R} connector at the base of the dry injector by a plate held between the captive nut and the sleeve. With the column outlet connector and the dry injector Glaslok^{R} connector secure the column is supported vertically in a closed basket (Plate 5) which can be easily dismounted orre-installed without manipulation of fragile components. The injector heater (Pye Unicam Ltd., Cambridge, England, No.717190) is mounted permanently on the injector assembly and injector heater and carrier gas inlet are directly compatible with gas flow controllers and proportional energy controllers integral to the chromatograph.

2.2.8. Detectors. Kaiser has stated that there are three requirements for detectors employed with open tubular columns: (1) Very small effective volumes; (2) Very high sensitivity; (3) Low time constant.[194] Ionization detectors and in particular flame ionization detectors have been recognized as particularly suitable in this respect.[197] Electron capture detectors have until recently not been effectively employed. An especially built ECD of low internal volume has been described which overcomes

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* Pye Unicam Ltd., Cambridge, England, Series 104 Gas Chromatograph.

** Scientific Glass Engineering Pty. Ltd., 657 North Circular Road, London, NW2 7AY, Cat. No. GCC/100.

the concentration dependent nature of the detector.[183] In this work flame ionization detectors were employed in the same manner as they might have been for packed columns and in all cases the performance was satisfactory.

2.3. Combined Gas Chromatography-Mass Spectrometry with Glass Open Tubular Columns.

2.3.1. Introduction. Gas chromatography is capable of separating, detecting and characterizing on the basis of retention behaviour very small quantities of a wide variety of organic compounds. Mass spectrometry provides definitive structural information on similar quantities of material. The dynamic coupling of the two instrumental systems while combining the practical problems of the constituent techniques provides a tool of unique power in the identification and quantification of the constituents of complex mixtures of synthetic and biological origin.

This coupling is complicated by the widely differing pressures at which the two instruments operate. The column inlet pressure of the chromatographic column is slightly above atmospheric pressure and the ion source of the conventional mass spectrometer at a pressure at or below 10^{-9} atmosphere. This problem has been solved by a variety of more or less complicated "separator" devices which serve the single-minded purpose of rectifying this pressure differential by divesting the column effluent of the maximum of carrier gas while transferring the maximum of solute to the ion source of the mass spectrometer. This variety has been comprehensively reviewed by Simpson[233] and it is proposed here to discuss those devices which have found application with open tubular columns.

The general requirements for a detector employed with open tubular columns (Section 2.2.8.) are equally valid for the ion source of the mass spectrometer. Whether employed as an ionization detector of greater (selected ion monitor) or lesser (total ionization monitor) specificity or to obtain "full scan" mass spectra the considerations remain broadly the same. The separator-ion source combination functions integrally to produce a signal specific to the instantaneous composition of the effluent of the chromatographic column. The electron impact ionization source of the type used on most instruments has a small effective volume, is highly sensitive in respect of signals obtained through the electron multiplier and associated amplifiers of the mass analyser. Response, apart from

the time required for the scanning of the complete spectrum , is nearly instantaneous. On purely instrumental and statistical grounds the quality of mass spectrum obtained improves with slow scanning. On the other hand, to obtain an unbiased mass spectrum of a component emerging from a gas chromatograph it is necessary to have a fast scan so that the partial pressure of the component in the eluate is as nearly constant as possible. Practical and theoretical considerations make this of particular importance with open tubular columns. In the first instance the generally narrower band width of the peak emerging from the open tubular column makes a rapid scan more important since the pressure of eluate is more transient. Secondly, in the broadest possible sense, within a given class, compounds of nearly identical chromatographic retention are on the whole likely to be similar in structure and exhibit similar mass spectra. The relative intensities of fragment ions are of increased significance in the identification of such compounds. High efficiency chromatography in g.c.m.s. makes the correct rendering of the mass spectra of particular importance.

Thus the suitability of the separator-ionization source unit as a chromatographic detector for open tubular columns is to a great extent the suitability of the separator device itself. Sensitivity must be maximized by the transference of the maximum amount of eluate to ionization source with the minimum of time lag and of mixing both of which impair chromatographic performance. Four types of interface have found application with open tubular columns.

(a) The permeable membrane separator. Organic molecules may be separated from carrier gas by the preferential permeation of a thin elastomer membrane by the organic molecules, whereby they pass into the source of the mass spectrometer. A device functioning on this basis was first reported by Llewellyn and co-workers.[65] The principal disadvantage of separators employing this principle is the degradation of chromatographic resolution produced by time lag in the absorption and desorption using silicone rubber membranes have been employed with open tubular columns by Black and co-workers[66] with the loss of 60 to 70 per cent of the available efficiency. Other devices employing the principle of differential transfer through a continuous barrier have not found application with high efficiency columns.

(b) The effusion separator. Watson and Biemann first introduced a molecular separator operating on an effusion principle.[234] This was later refined by Markey[54] for application with higher molecular

weight materials. The required pressure drop to mass spectrometer vacuum is effected by capillary entrance and exit restrictions on either end of a porous fritted glass tube within a vacuum envelope. Under the molecular flow conditions, within the passages of the ultrafine (1μm average pore size) porosity glass frit, flow is inversely proportional to molecular weight and directly proportional to the difference in partial pressures across the frit. The factors favour the effusion of lighter carrier gas and the enrichment, in the effluent from the device, of the higher molecular weight components. Völlmin and co-workers employed this type of separator with open tubular columns for the analysis of the constituents of cigarette smoke.[235] The degradation of chromatography efficiency is the major inadequacy. The chromatographic efficiency of the system was 70 per cent of that available from the column.[161]

(c) The jet-orifice separator. The principle of the separation jet[236] was first utilized as a diffusive means of the preferential removal of helium from column effluent by Ryhage.[237] In the device column effluent is forced to a convergent nozzle from which the gaseous mixture emerges as an expanding jet. Directly opposite and in line with this nozzle is a second opening. Because of lower forward momentum and greater diffusivity the lighter carrier gas molecules are removed into an evacuated peripheral volume, thus enriching the stream of material passing into the second opening in respect of the heavier components. Optimal adjustments of the dimensions of the orifices and their separation, as well as their arrangement in single and two stage units,[67,237] serve to adapt the concept to a variety of packed and open tubular column[238] applications. An adjustable version offers the possibility of optimum performance with both types of columns.[239] Patent protection has generally limited this all metal separator to a single gas chromatograph-mass spectrometer instrument. However, a single stage glass jet-orifice separator has appeared as interface in quadrupole and magnetic sector mass spectrometers. The now well known fixed jet two stage jet separator has been successfully adapted for application with open tubular columns either by removing the first jet[203,240,241] or by supplying additional gas to the separator after the column to make up the total rate to that demanded for efficient operation to the fixed jet device.[227,242]

(d) Direct coupling. The first connection of a gas chromatograph

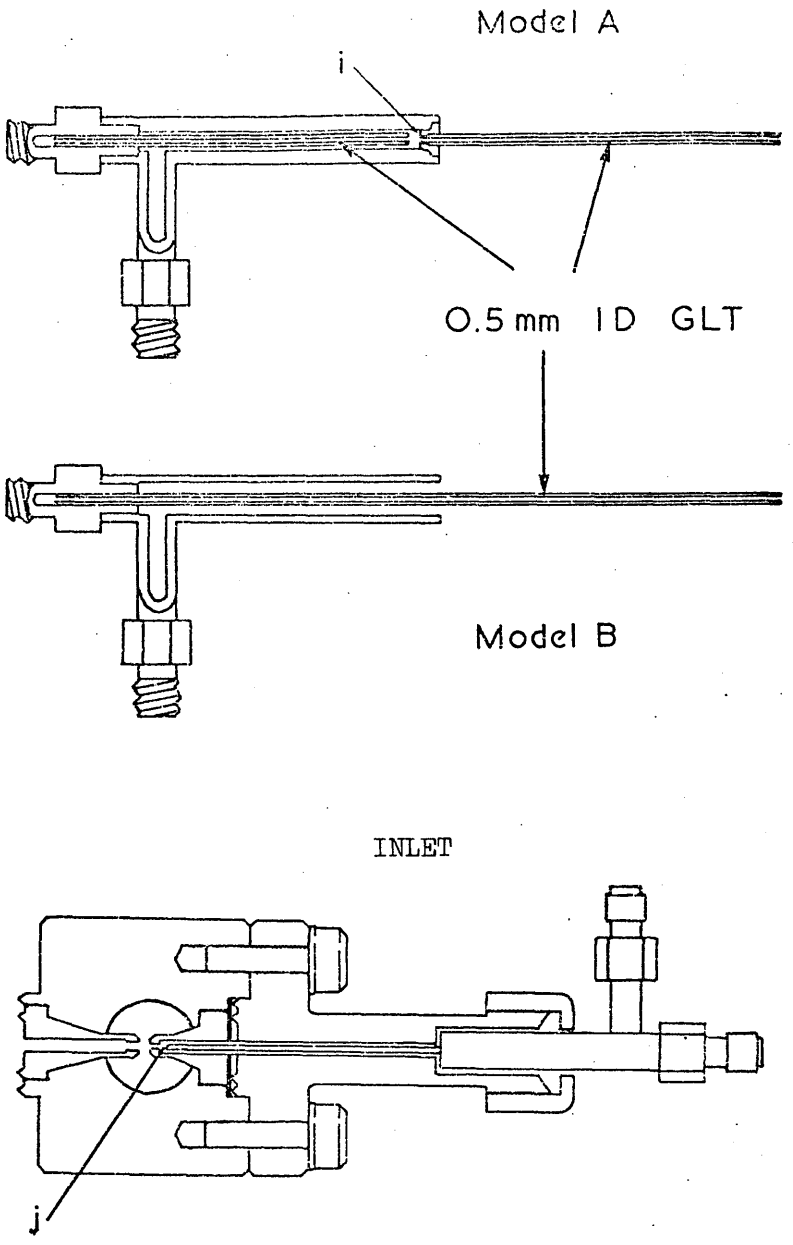

Figure 2.10. Make-up gas adapters employed to increase open tubular column flow rates to that required by Becker-Ryhage molecular separator. Make-up adapter Model A the column effluent and make-up flows combine within the device at point (i). In make-up adapter Model B the column effluent is led directly to a point just before the first jet of the separator (j) where it is combined with make-up flow.

to a mass spectrometer was made by Holmes and Morrell.[243] Here the connection was by means of a splitter system which admitted 1 per cent of the column effluent to the ion source of the instrument. Shortly thereafter low molecular weight compounds were studied by Gohlke by directly admitting a 1ml/min total column effluent from a metal open tubular column to a time-of-flight mass spectrometer.[244] From the splitter of Holmes and Morrell onwards through the variety of separators devised to interface columns to mass spectrometers, all systems have failed to match the simplicity of that early concept of Gohlke. This was the result of compromises required in matching total column flow rates to the limitations of pumping capacity of existing vacuum systems. With advances in vacuum technology, progress has been made in returning to that earlier ideal.[245,246] The problems posed by open tubular columns are smaller by virtue of their generally lower volumetric flows. Henneberg and co-workers have demonstrated the advantage of a direct inlet, via a narrow platinum capillary, even for an instrument of relatively low pumping capacity.[247] An "open split" system was employed allowing a simple and rapidly interchangeable connection between the g.c. and the m.s.

The simplicity of direct coupling, however it is accomplished in detail, eliminates many factors causing loss of column resolution, such as absorption, thermal degradation and mixing. With the advent of thermally stable glass open tubular columns, direct coupling to the mass spectrometer has been used to good effect in the analysis of complex mixtures of compounds of biological origin.[228,232,248]

2.3.2. The make-up adaptor. The mass spectrometer employed in the work, an LKB 9000, is a low resolution, rapid magnetic scanning, single focusing instrument equipped with a two-stage stainless steel jet-orifice separator of the Becker-Ryhage design. The pumping capacity of the vacuum system requires an enrichment factor from the separator of 100. The fixed dimensions and configuration of the separator were designed to operate in conjunction with packed columns at a flow rate of 30ml/min of helium, the efficiency being 60 to 70 per cent. As the flow through the separator is critical to separator performance, measures must be taken to modify the separator for use with open tubular columns; alternatively gas may be added to column effluent before the separator to make up the flow to the required rate. The latter procedure was chosen to maintain the

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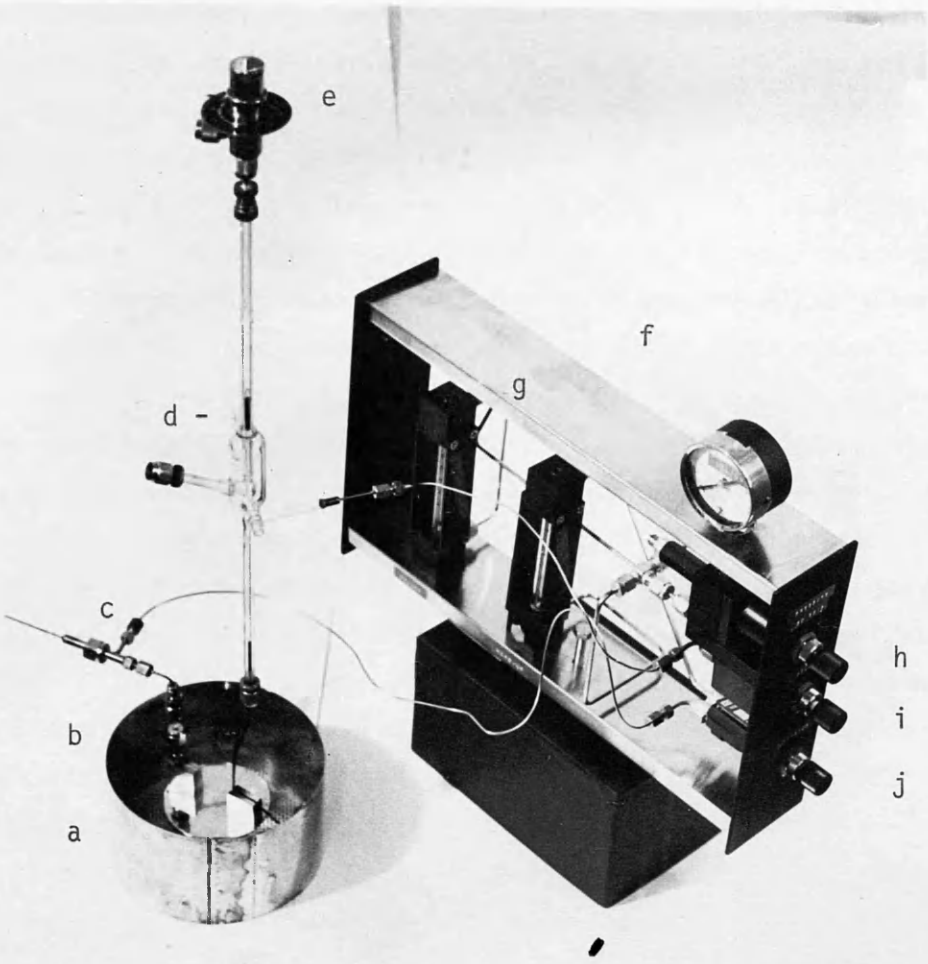

Plate 6 Glass open tubular g.c.-m.s. assembly. (a) Support basket with glass-graphite GLT connectors, (b) glass restrictor, (c) make-up gas adaptor, (d) glass dry injector, (e) metering valve, (f) gas control manifold, (g) flow meters, (h) secondary pressure control, (i) injector flow control, (j) make-up flow control.

flexibility of the g.c.-m.s. instrument which is also required for a wide variety of packed column applications. A modular system was devised using the make-up gas concept to allow the rapid and uncomplicated exchange of packed for open tubular columns.

Figure 2.10. shows two versions of the device constructed to add make-up gas to column effluent. Both models are constructed in stainless steel. The tubing for conduction of column effluent is 0.5mm i.d., 1/16" o.d. glass lined metal tubing (GLT) silver soldered in appropriate positions. Connections from column outlet connectors and gas inlet to the make-up adapter are Pye 2 B.A. tube connectors. The external diameter of the device allows connection to the separator with heat-resistant front ferrules (Packard Limited, Caversham, Berks.) in the Swagelok^{R} type union in the fashion as packed columns. It is necessary that the flow be increased to that required for optimum separator performance in a laminar fashion, without impairing the column performance.

In model A, column effluent is admitted at the Pye connector on the long axis of the device and channelled via glass lined metal tubing to a point 2mm from the exit tube orifice (i), where it is combined with gas delivered through the side arm. A second length of glass lined metal tubing delivers the combined stream to a point 1mm before the first jet of the Becker-Ryhage separator (j). Model B differs in that the column effluent is delivered directly to point (j) where it is combined with gas passing down the annular space between the bore of the separator connector assembly and the make-up adaptor. Immediately the streams combine they pass into the first jet of the separator.

2.3.3. The combined open tubular g.c.-m.s. assembly. The make-up and flow system are constructed according to Linnarson[249] and are combined with the dry injector and a column support basket which fits directly into the oven of the LKB 9000. The modular arrangement of components (Plate 6) allows the column to be installed in the support basket (a) with its integral connectors outside the instrument. When the mass spectrometer becomes available the support basket is inserted into the column oven, the make-up adaptor (c) installed, the dry injector (d) connected to the column inlet connector and all connections to the gas control system (f) made (Plate 7). The column and make-up flows are reduced to minimum levels and the separator is pumped down in the same way as with packed columns. The column and make-up flows are established and the system is tested for

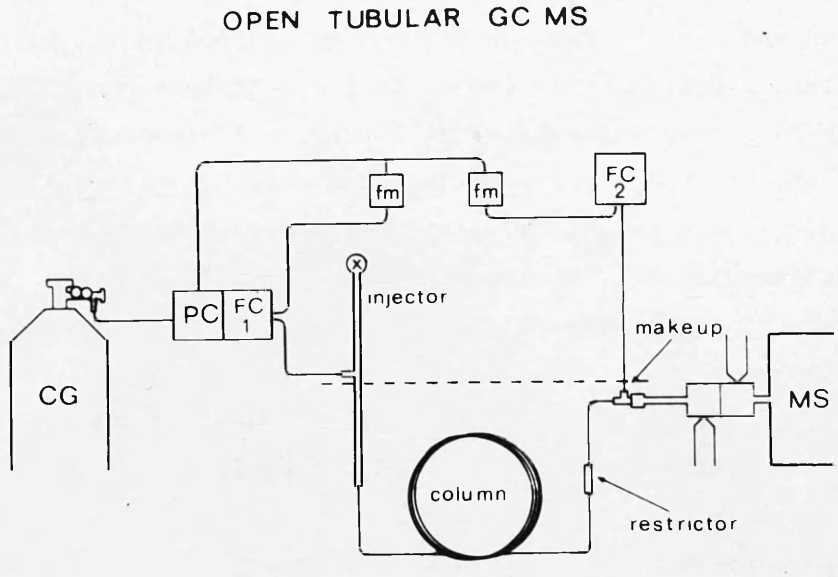

Fig. 2.11 Schematic of pneumatic system of glass open tubular g.c.-m.s. assembly.
P.C. = secondary pressure controller, Fm = Rotameter Flow meters, FC1 & FC2 = flow controllers, CG = high purity helium, MS = LKB 9000

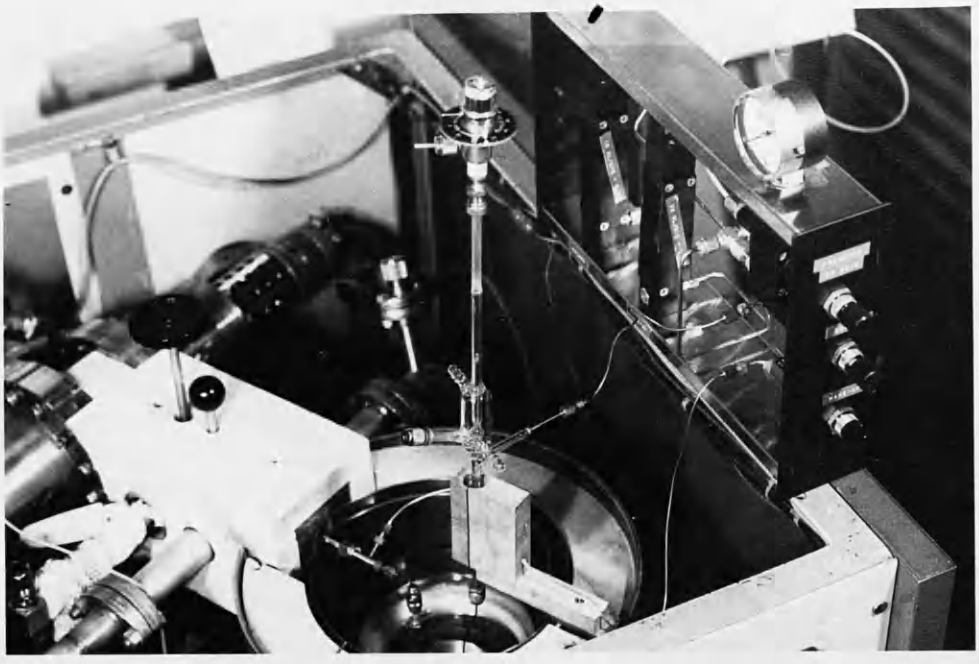

Plate 7 Glass open tubular g.c.-m.s. assembly installed in LKB 9000

leaks. The column oven is brought to operating temperature and the instrument put into operation in not substantially longer time than is required for the exchange of packed columns.

Figure 2.11 represents the pneumatic system which provides carrier gas and make-up gas for the column coupled to the mass spectrometer. Helium is delivered to the unit by a conventional cylinder head pressure regulator at 60psig. A secondary pressure regulator (Model 8601, Brooks Instruments Ltd., Stockport, Cheshire) delivers gas at a constant 50psig through flow meters (Model 1350, Brooks Instruments Ltd., Stockport, Cheshire) which in turn regulate the delivery of gas to the injector and the make-up adaptor. All connecting tubing in the manifold is 1/8" stainless steel with 1/8" Swagelok^{R} unions at all units. All connecting tubing is 1/16" stainless steel tubing (i.d. 0.030") equipped with Pye 2 B.A. tubing connectors.

Column flow with outlet pressure at atmospheric is a function only of inlet pressure as determined by the vent restrictor on the dry injector. With the make-up system the situation is somewhat more complex. The outlet pressure of the column within the make-up adaptor is at some unspecified sub-atmospheric level determined by the flow of the column and make-up and the flow through the first jet into the separator. It was found that at temperatures below 150^{o}C and with columns less than 30m in length the pneumatic resistance of the open tubular column was insufficient to maintain flow rates at the required levels even with no vent restriction at the injector, i.e. inlet pressure of one atmosphere. It was found necessary to include a flow restrictor in series with the column after the column outlet. This was incorporated in the column outlet connector and consisted of a glass capillary 4cm in length and 0.08mm internal diameter (Veridia tubing: Chance Brothers Ltd., Malvern Link, Worcestershire) mounted in a pair of 316 stainless steel Swagelok^{R} 1/8" to 1/16" reducing unions by 1/8" graphite front ferrules (Scientific Glass Engineering Pty. Ltd., 567 North Circular Road, London, NW2 7AY). The narrow passages of the union are drilled out to 1/16" so that the two sections of glass lined metal tubing may butt directly against the capillary opening in the glass restrictor. This produces a dead volume-free, all-glass restriction which decreases the conductance of the system and makes the flow controllable by means of the injector vent restrictor. Without any direct access to the column outlet under operating conditions,

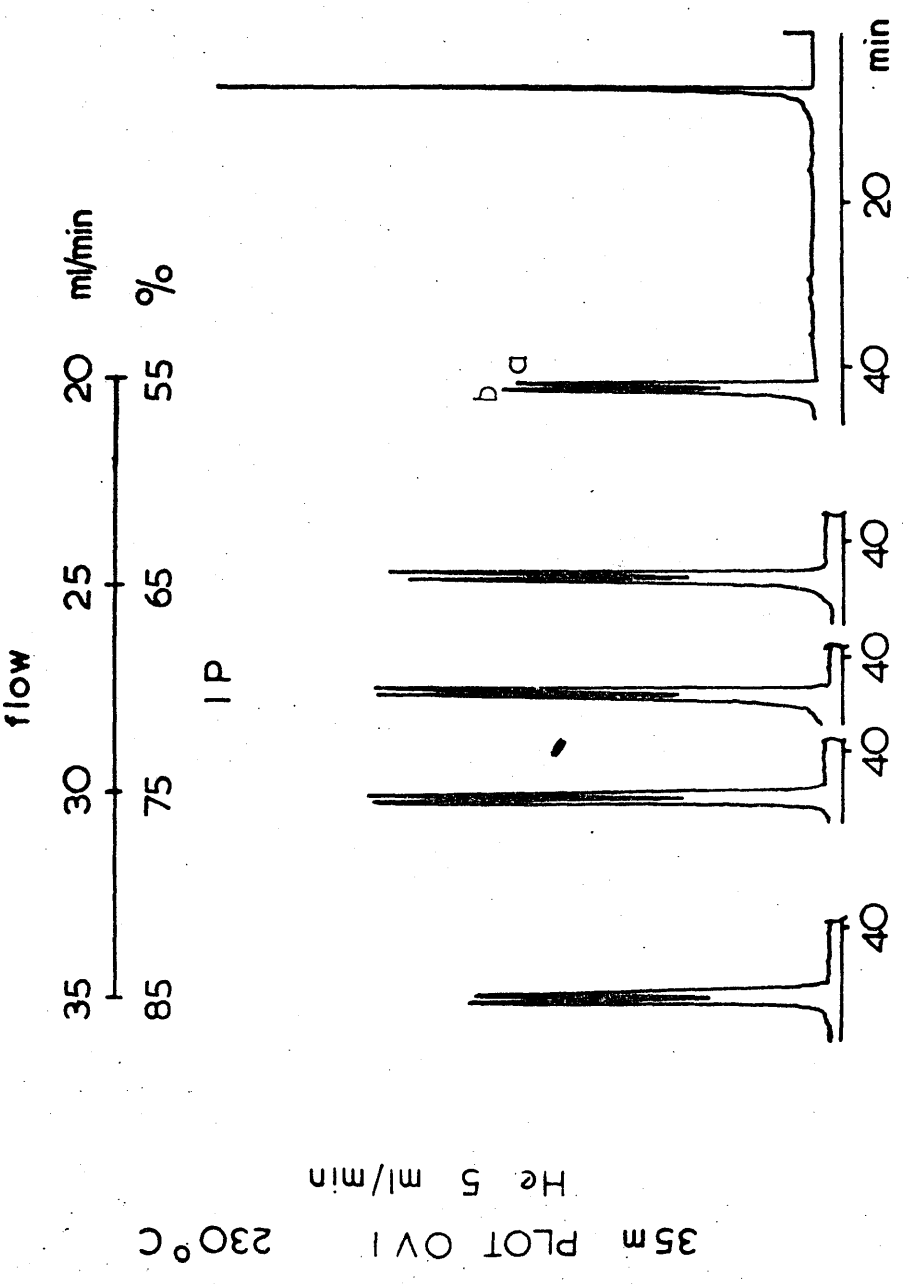

Figure 2.12. Effect of total separator flow (column + make-up) on separation and yield of 0.5µg each of 5-androsten-3β-ol TMS ether (a) and 5α-androstan-3β-ol TMS ether (b) LKB 9000 TIC chromatogram. 35m Silanox-type glass (PLOT) OV-1 column and restrictor at 230°C, injector heater at 250°C, with column flow rate at 5ml/min.

the flow rates are measured as the difference in injector and vent flows at column operating temperature. The injector flow is determined before connection to the injection system and the vent flow measured with a bubble flow meter after the desired conditions have been established.

2.3.4. Make-up system function and separator performance. Figure 2.12. shows a composite of five chromatograms illustrating the effect that the total flow, as controlled by the flow of make-up gas, exercises on the efficiency and yield of the Becker-Ryhage separator. A 35m Silanox-type glass (PLOT) OV-1 column with restrictor was installed in the LKB 9000 in the normal fashion and the column oven raised to 230°C with column flow at 5 min. A test solution of 0.5mg each of 5-androsten-3β-ol and 5α-androstan-3β-ol (Medical Research Council Steroid Reference Collection) was trimethylsilylated in the conventional manner and aliquots of the solution of TMS ethers were applied to the dry injector and chromatographed at total separator flows, estimated by pressure of the first stage of the separator, from 20 to 35ml/min. The total flow showed no perceptible effect on the separation of the 5α-Δ^5 sterol pair. However, separator yield showed a plateau between 25 and 30ml/min total flow. Based on this, standard conditions were established for total flow of 27.5ml/min. This produced a pressure at the first stage of the separator of 6×10^{-2}torr (inlet Pirani gauge Range II:70% full scale deflection). The pressure of the second stage of the separator was less than 2×10^{-4}torr (inlet Pirani gauge Range II: less than 2% full scale deflection) and the ionization source pressure was 2×10^{-6}torr (ion source Penning gauge).

It is interesting to note the gradual and stepwise increase in the retention time of the test substances with increasing separator flow. The pressure drop along the column, that is, the column flow rate, is a complex function of the inlet pressure (controlled by injector restrictor) and the pressure produced by the combination of column and make-up flows and of the flow to the first stage of the separator. This phenomenon corresponds to the decrease in column flow (i.e. pressure drop along the column) resulting from an increase in the outlet pressure of the column with increased make-up flow.

2.3.5. Chromatographic performance of the system. The effect of operating the outlet of an open tubular column under vacuum has been

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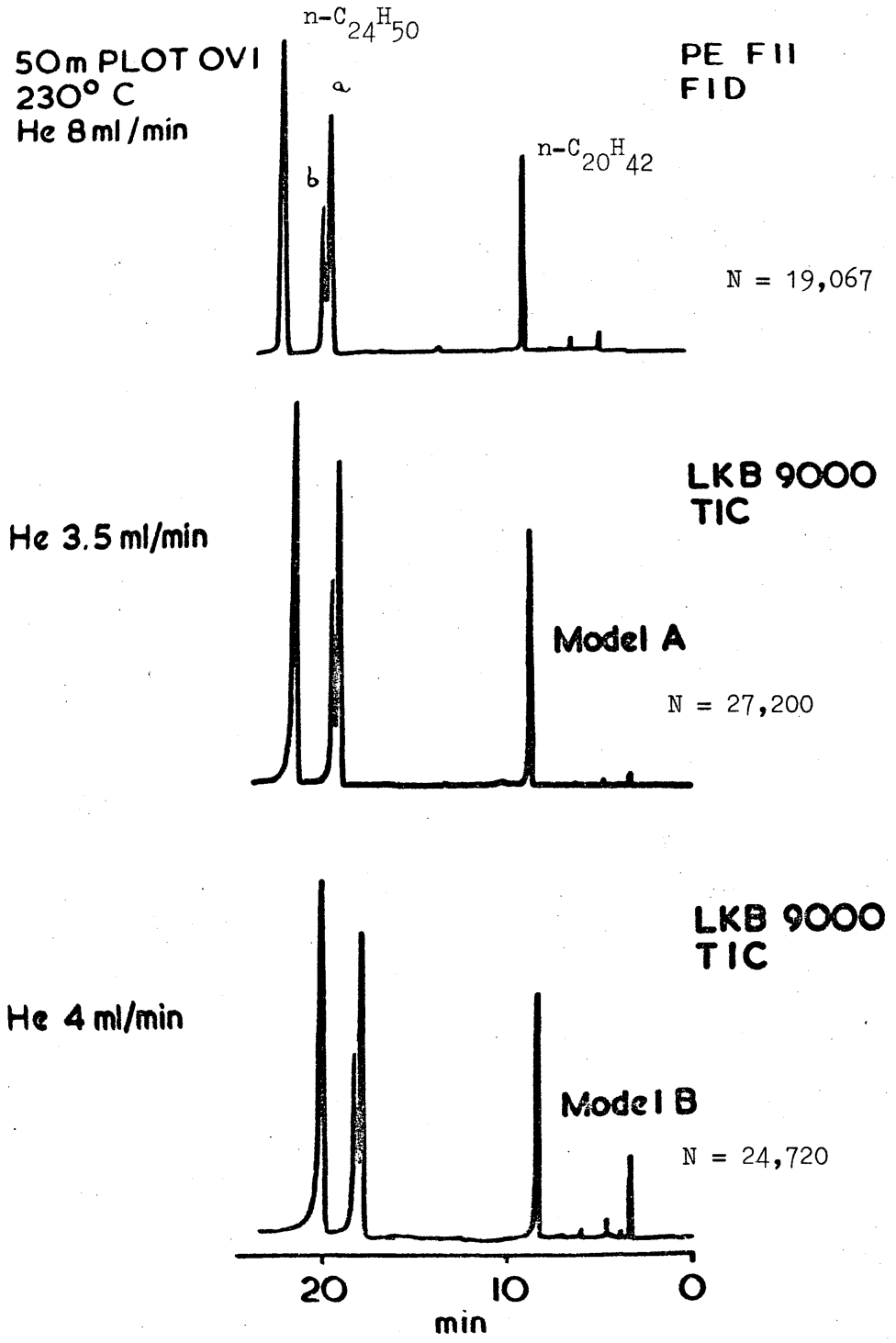

Figure 2.13. Comparison of performance of 50m Silanox-type (PLOT) OV-1 column in separation of 5-androsten-3β-ol TMS ether (a) and 5α-androstan-3β-ol TMS ether (b) installed in PE F11 (FID) and LKB 9000 with make-up adapters Model A and Model B. Number of theoretical plates (N) calculated from n-$C_{24}H_{50}$ by Equation 2.1.

explored by Varadi and Ettre[250] and by Teranishi and co-workers.[251] The operation under such conditions is advantageous with similar or enhanced efficiencies and shortened analysis times. Novotny has observed that with the column outlet at reduced pressure in a jet separator there is a shift in the optimum linear gas velocity towards higher values which is useful in reducing analysis times at no penalty of column efficiency.[240] Figure 2.13. compares the separation of 5-androsten-3β-ol TMS ether and 5α-androstan-3β-ol TMS ether obtained on a 50m Silanox-type glass (PLOT) OV-1 column installed alternately in a Perkin-Elmer Model F11 gas chromatograph equipped with flame ionization detector (FID) and in the LKB 9000 (without column outlet flow restrictor). Comparison of flow conditions between the FID (with column outlet pressure at atmospheric) and the mass spectrometer is difficult. In this instance, carrier gas velocities were adjusted to produce identical retention times for n-eicosane (n-$C_{20}H_{42}$) at 230°C. The make-up adapters differed slightly in their behaviour in respect to column flow rates. The column inlet pressure for Model A was at atmospheric pressure and for Model B was slightly over atmospheric (injector valve open and slightly closed respectively). Flow rates were measured as the difference between the injector flow, measured before connection to the injector, and vent flow at column operating temperature. Column flow for the FID was measured at column operating temperature by a bubble flow meter.

A test solution of 0.1mg 5-androsten-3β-ol and 0.5mg 5α-androstan-3β-ol was prepared and derivatised in the normal way and the TMS ethers were taken up in 1.0ml of Nanograde hexane. 1.0µl of this solution and 1.0µl of a solution of standard n-alkanes (n-$C_{20}H_{42}$ and n-$C_{24}H_{50}$ at approximately 1.0µg/µl) were injected in the normal way using the dry injector on 50m Silanox-type (PLOT) OV-1 column installed alternately in each of the instrumental configurations. Figure 2.13. compares the results. The calculated number of theoretical plates (Equation 2.1.) showed a substantial improvement in the g.c.-m.s. installation. The separation of the 5α-Δ^5 pair shows no substantial change. The peak shape obtained on the LKB 9000 showed a pronounced "stiletto" shape when compared with the more normal shape observed on the FID trace. There is some tailing on the TIC traces but in general terms the usefulness of the two systems seems comparable.

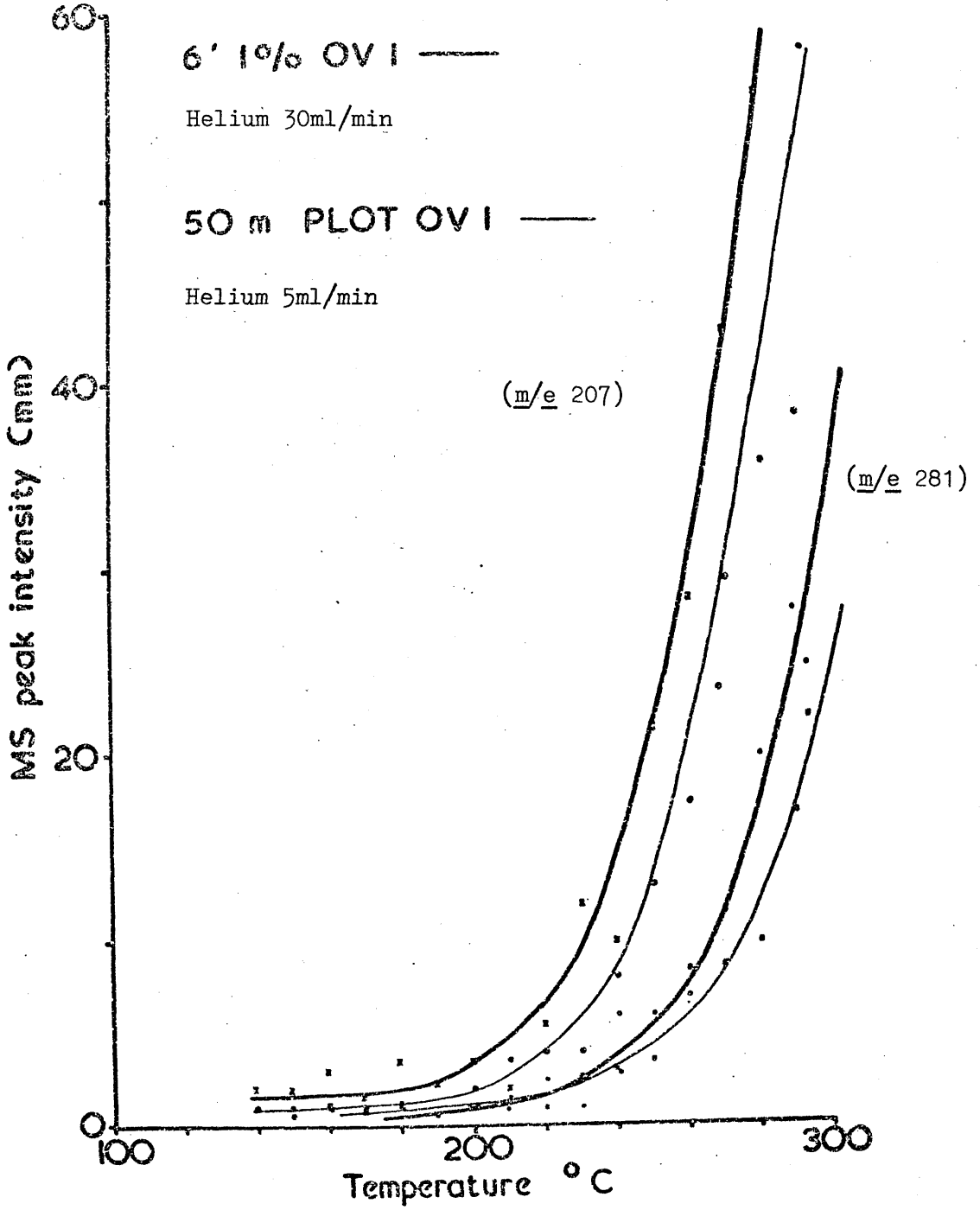

Figure 2.14. Comparison of "background" of OV-1 from packed and open tubular columns measured as peak height at m/e 207 and m/e 281 at 22.5eV (2nd trace of uv recorder). Columns under normal conditions programmed from 120°C to 300°C at 1°C/min. LKB 9000 (multiplier sensitivity 250, 3.1kV) scanned at 10°C intervals.

2.3.6. Column background. Volatile materials "bleeding" from stationary phases coated on column packings or on the bore of open tubular columns are of particular significance when these columns are employed for gas chromatography-mass spectrometry. At high temperatures a range of characteristic ions form the bulk of this total "background" and this factor is of the greatest significance in the selection of stationary phase, the maximum allowable temperature and the loading of phase which may be used for g.c.-m.s. analysis. Figure 2.14. shows a comparison between the backgrounds obtained from well conditioned packed and open tubular columns. The packed column (6 ft. x 3.5mm i.d. glass spiral column packed with 1% OV-1 on 100-120 mesh Gas Chrom Q) and the open tubular column (50m x 0.5mm Silanox-type (PLOT) OV-1) had a long history of successful use at temperatures up to 300°C. Each was installed in the LKB 9000 and conditioned overnight at 280°C. The columns were cooled to 100°C and allowed to stabilize and then programmed at 1°C/min to 300°C. The mass spectrum for the mass region m/e 200 to m/e 300 electron energy 22.5eV, multiplier sensitivity 250 (3.1kV) was recorded at 10°C intervals through the programme and the height of the m/e 207 and m/e 281 ions characteristic of the methylsilicone stationary phase were measured on the second galvanometer oscillographic recorder trace. From Figure 2.14. it can be seen that the background level for the open tubular column generally remains at 50 per cent of the packed column level. This conforms to the observation of other workers employing open tubular columns but the advantage is modest compared to the reduction of 95 per cent reported by Luyten.[225]

2.3.7. Improved mass spectral sensitivity with open tubular columns. With the ionization source of the mass spectrometer, in common with the flame ionization detector and other ionization detectors, it is not the absolute amount of a particular sample component that determines its detectability. It is the amount of sample reaching the detector in unit time which is important, the sensitivity being properly expressed as g/sec or moles/sec. That is these detectors are concentration rather than mass sensitive. Thus with a detector of given sensitivity if a peak is "sharpened" then the minimum detectability and overall sensitivity of the system is improved. For a given column this may be achieved by increasing the flow rate or by temperature programming. By the exchange of columns for one of greater efficiency this increase in dynamic performance can be

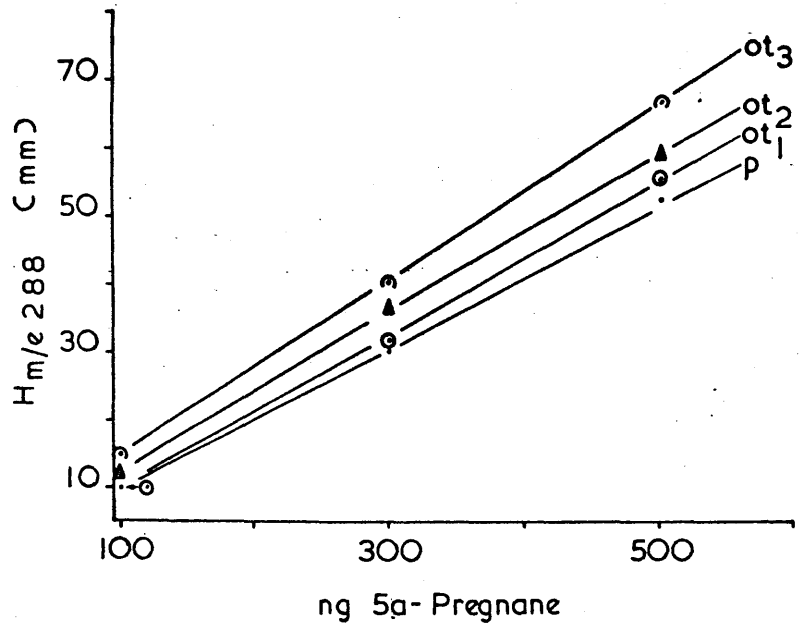

Figure 2.15. Comparison of the mass spectral sensitivity to 5α-pregnane with a packed and open tubular column. The sensitivity was measured as the height in mm of m/e 288 on the second galvanometer trace of the uv recorder. MS operating conditions: electron energy 70eV, multiplier sensitivity 240 (2.95kV), source pressure 2 x 10^{-5}torr. The chromatographic performance of each system is tabulated below for column temperature 200°C and helium flow rate sufficient to produce the t_r indicated.

Diagram reference	column	t_r 5α-pregnane	number of theoretical plates*
p	6ft 1% OV-1	4.6min	2,400
ot_1	5m PLOT OV-1	11.4min	30,300**
ot_2	"	9.2min	10,600
ot_3	"	7.5min	7,600

* Calculated for n-$C_{24}H_{50}$

** Flow approximately 5ml/min, same column installed in Pye 104 gas chromatograph, T = 200°C, He5ml/min: n = 32,300 for n-$C_{24}H_{50}$.

achieved and it is here that open tubular columns provide an advantage for sensitivity as well as resolution over packed columns.

The sensitivity of the LKB 9000 for the total ionization current (TIC) chromatographic detector and the mass spectrum (MS) recording system is specified by the manufacturer for signals produced by 0.1µg of 5α-cholestane chromatographed on a 1% SE-30 packed column with flow rate at 30ml/min at such a temperature that the peak emerges in 10 ± 1 minutes. The TIC peak produced under these conditions (electron energy 22.5eV, TIC amplifier sensitivity at position 9) is specified to give a peak height of 50 ± 25mm. For MS recording system electron energy 70eV, multiplier sensitivity 222 (2.7kV) is specified to/a peak height of 25 ± 15mm for the molecular ion of 5α-cholestane (m/e 372). While this specification is designed primarily for the maintenance and adjustment of the instrument in principle it can also serve to compare the effect on instrument sensitivity of exchanging packed for open tubular columns.

Figure 2.15. shows a comparison of the mass spectral sensitivity performance of the LKB 9000 for a packed and an open tubular column. The mass spectral sensitivity was within the above mentioned specification. Aliquots of a standard solution of 5α-pregnane (Aldrich Chemical Company, Milwaukee, Wis., C7420) were injected on a 6ft 1% OV-1 glass column, 3.5mm i.d., coated on 100-120 mesh GCQ (Applied Sciences Laboratories) and a 50m x 0.5mm Silanox-type glass (PLOT) column prepared according to the procedures outlined previously (Section 2.1.2.). The chromatographic conditions were T = 200°C and helium flow rate at 30ml/min for the packed column and 5ml/min for the open tubular column (plus two higher rates). 5α-pregnane was chosen as a standard to minimize problems of long retention times and the sample range of 100-500ng chosen to avoid amounts which were either so small as to be comparable to any conceivable absorption effects or so large as to affect the shape of peaks emerging from the open tubular columns.

From Figure 2.15. it can be seen that the two systems compare favourably with respect to MS sensitivity. The resolution advantage with the open tubular column, 'sharper peaks', more than counters the liability of somewhat longer retention times. If the flow rate in the open tubular columns is increased with the corresponding decrease in chromatographic efficiency this margin may be further exploited. Under these conditions the mass spectrum of 25ng of 5α-pregnane was complete and usable even in the least advantageous

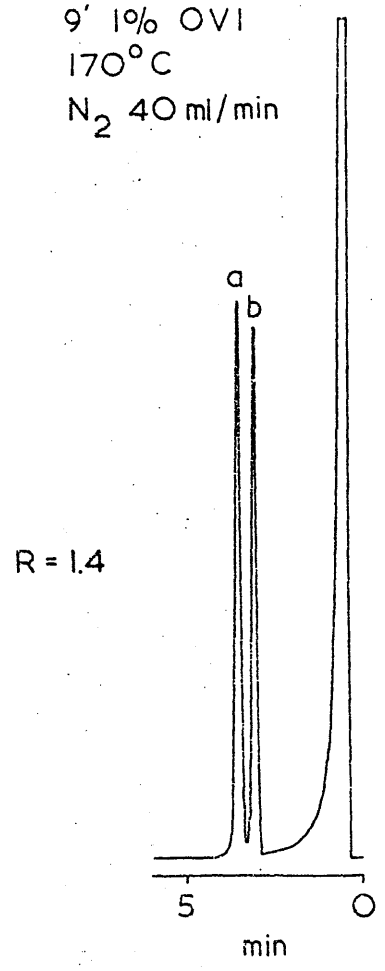

Figure 2.16. Packed column separation of 5α-androst-16-ene (b) and 5α-androstane (a).

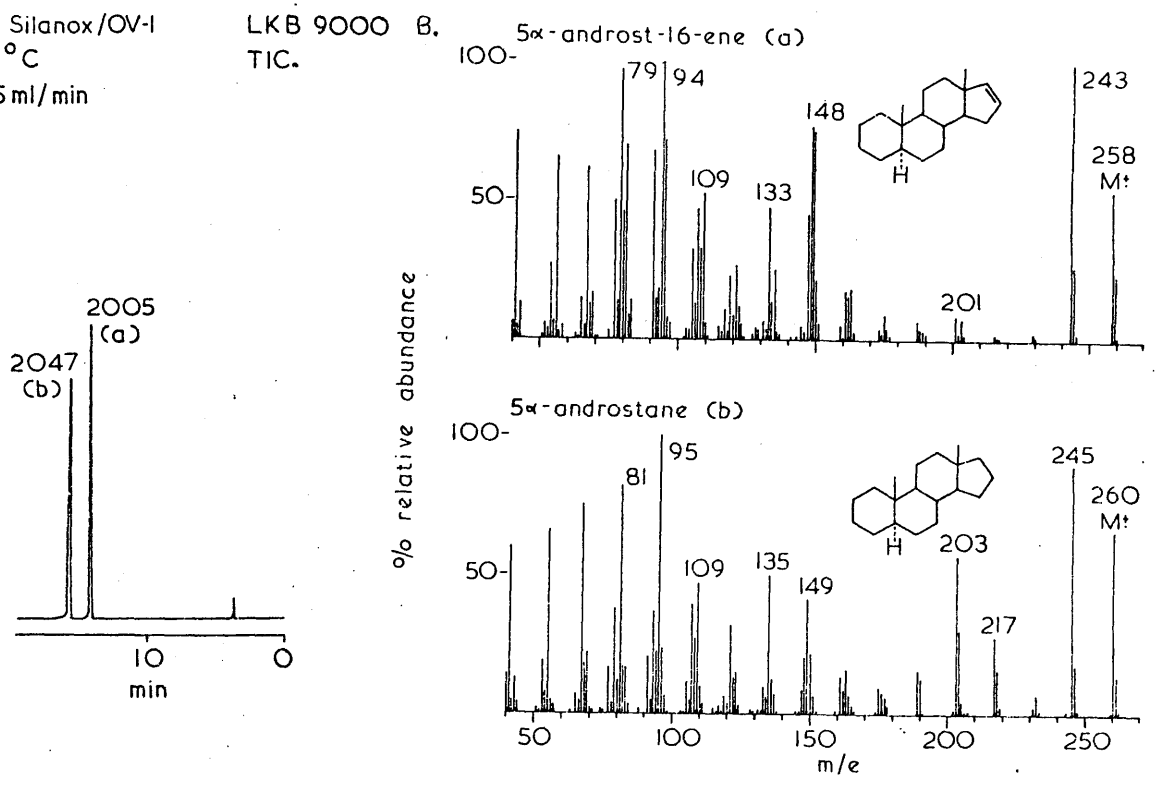

Figure 2.17. Total ionisation current (TIC) chromatogram (left) and electron impact (EI) mass spectra (right) of a mixture of 5α-androst-16-ene (a) and 5α-androstane (b). Retention indices appear on the chromatogram. Chromatographic conditions: 50m x 0.5mm i.d. Silanox-type (PLOT) OV-1 glass column, installed with make-up gas adaptor in the LKB 9000, temperature 230°C, helium carrier gas 4ml/min. Mass spectrometer conditions: source temperature 270°C, electron energy 70eV.

instance of optimum chromatographic resolution with the open tubular column.

So it can be seen that chromatographic efficiency may be traded for shortened analysis time and an increase in overall sensitivity. This may be achieved by increased flow rate or by shortening the overall column length. Under these circumstances it is possible to obtain chromatographic efficiencies equivalent to packed columns with very significant increases in the "sharpness" of the peaks eluted. Ettre has pointed out in a theoretical treatment of the question that if the chromatographic efficiency of an open tubular column were reduced to that of a conventional packed column by reducing the column length, the increase in response realized for bulk sensitive detectors would be tenfold.[252] Such potential bears much future attention.

2.3.8. Three trial separations.

(a) Separation of 5α-androstane and 5α-androst-16-ene. 0.5mg of 5α-androstane (Ikapharm, Ramat-Gan, Israel) and 0.5mg of 5α-androst-16-ene* were taken up in 0.5ml of Nanograde hexane. 1μl of this solution was co-injected with n-alkane standards ($n\text{-}C_{20}H_{42}$ and $n\text{-}C_{22}H_{46}$) on a packed column (9ft. 1% OV-1 installed in Perkin-Elmer Model 881) at 170°C and on an open tubular column (50m Silanox-type (PLOT) OV-1 installed in LKB 9000) at 230°C. Kováts retention index values[25,253] were calculated and are shown in Table 2.3.

Table 2.3. Kováts retention index values for 5α-androstane and 5α-androst-16-ene

Compound	Packed column	Open tubular column
	$I^{170°C}_{OV-1}$	$I^{230°C}_{OV-1}$
5α-androstane	1976	2047
5α-androst-16-ene	1937	2005

Figure 2.16. shows the separation achieved on packed column (R = 1.4) for the retention index difference of 39 Kováts units. Figure 2.17A shows the improved separation obtained on open tubular

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* Sample by courtesy of Dr. G.F. Woods, Organon Research Laboratories, Newhouse, Lanarks.

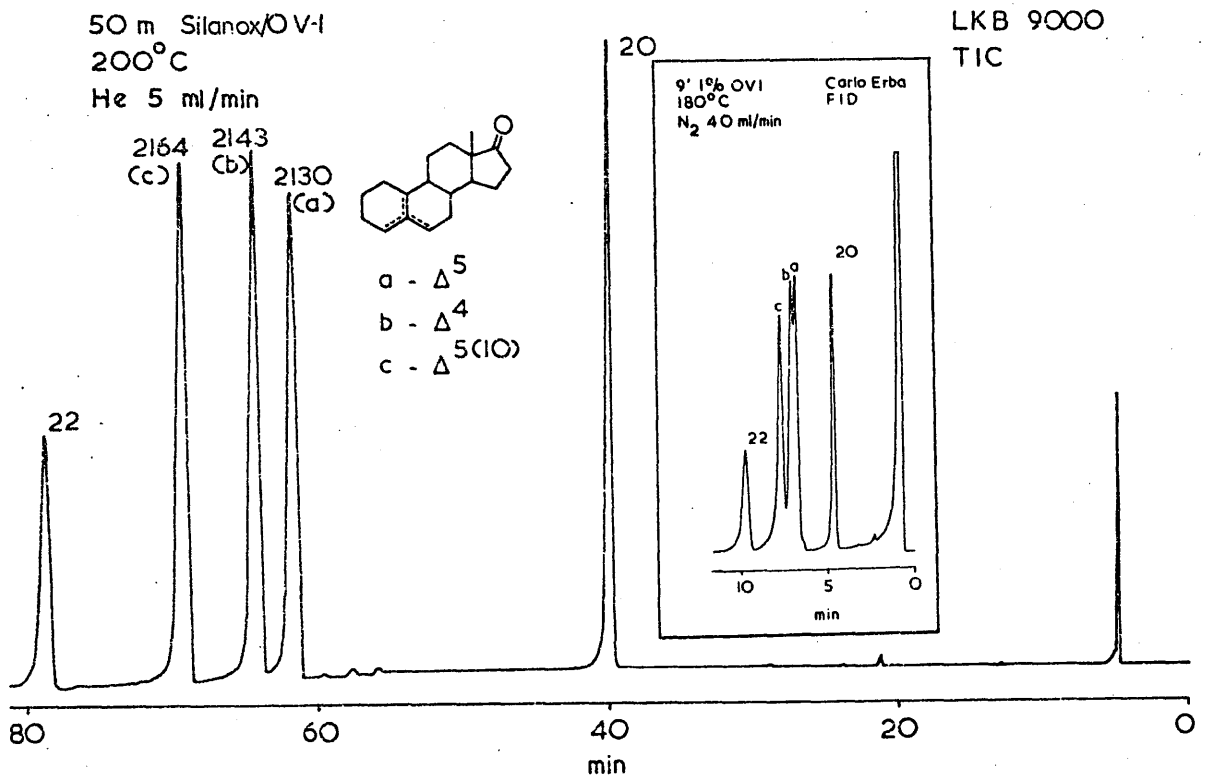
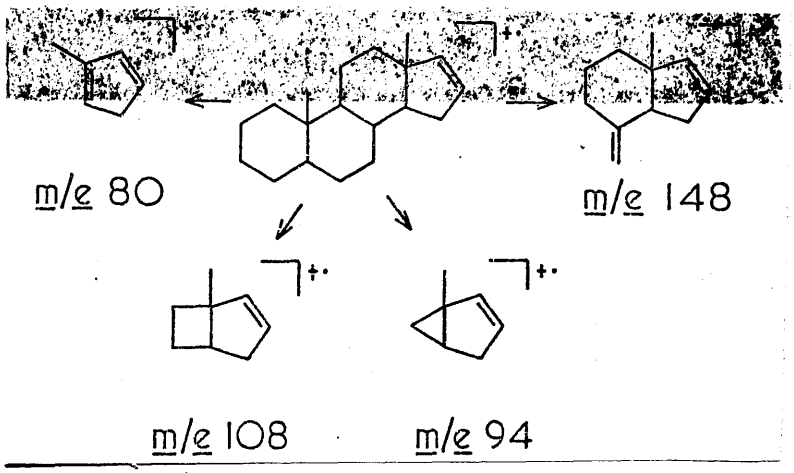


Figure 2.18. Total ionisation current (TIC) chromatogram of the separation of a mixture of estr-5-en-17-one (a), estr-4-en-17-one (b) and estr-5(10)-en-17-one (c). C_{20} and C_{22} n-alkanes are included as co-injected standards, and retention indices appear on the chromatogram. Chromatographic conditions: 50m x 0.5mm i.d. Silanox-type (PLOT) OV-1 column installed with make-up gas adaptor in the LKB 9000, temperature 200°C, helium carrier gas 50ml/min. Inset packed column chromatogram of the same mixture demonstrates the advantage of the more efficient open-tubular column.

column installed in LKB 9000. A retention index difference of 42 Kováts units was sufficient to effect complete resolution (R = 5.4). The mass spectrum of each component was obtained (electron energy 70eV, multiplier sensitivity 190 (2.2kV). These are shown in Figure 2.17B. The mass spectrum of the sterane was consistent with data recorded elsewhere.[254] The mass spectrum of the olefin shows distinctive fragmentation affording several even-mass ions (m/e 56,80,94,108,148) in high abundance. Possible sources of some of these are depicted below in Scheme 2.1.



Scheme 2.1.

(b) Separation of three estrenones.* 0.5mg each of estr-5-en-17-one, estr-4-en-17-one and estr-5(10)-en-17-one were combined and taken up in 0.5ml of Nanograde hexane. 1µl of this solution was co-injected with n-alkane standards (n-$C_{20}H_{42}$ and n-$C_{22}H_{46}$) on a packed column (9ft. 1% OV-1 installed in a Perkin-Elmer Model 881) at 180°C and an open tubular column (50m PLOT OV-1 installed in LKB 9000) at 200°C. The Kováts retention indices were calculated and appear in Table 2.4.

Table 2.4. Kováts retention index values for estrenones.

Compound	Packed column $I_{OV-1}^{180°C}$	Open tubular column $I_{OV-1}^{200°C}$
estr-5-en-17-one	2101	2130
estr-4-en-17-one	2115	2143
estr-5(10)-en-17-one	2138	2164

* Samples by courtesy Dr. G.F. Woods, Organon Research Laboratories, Newhouse, Lanarks.

facing page 74.

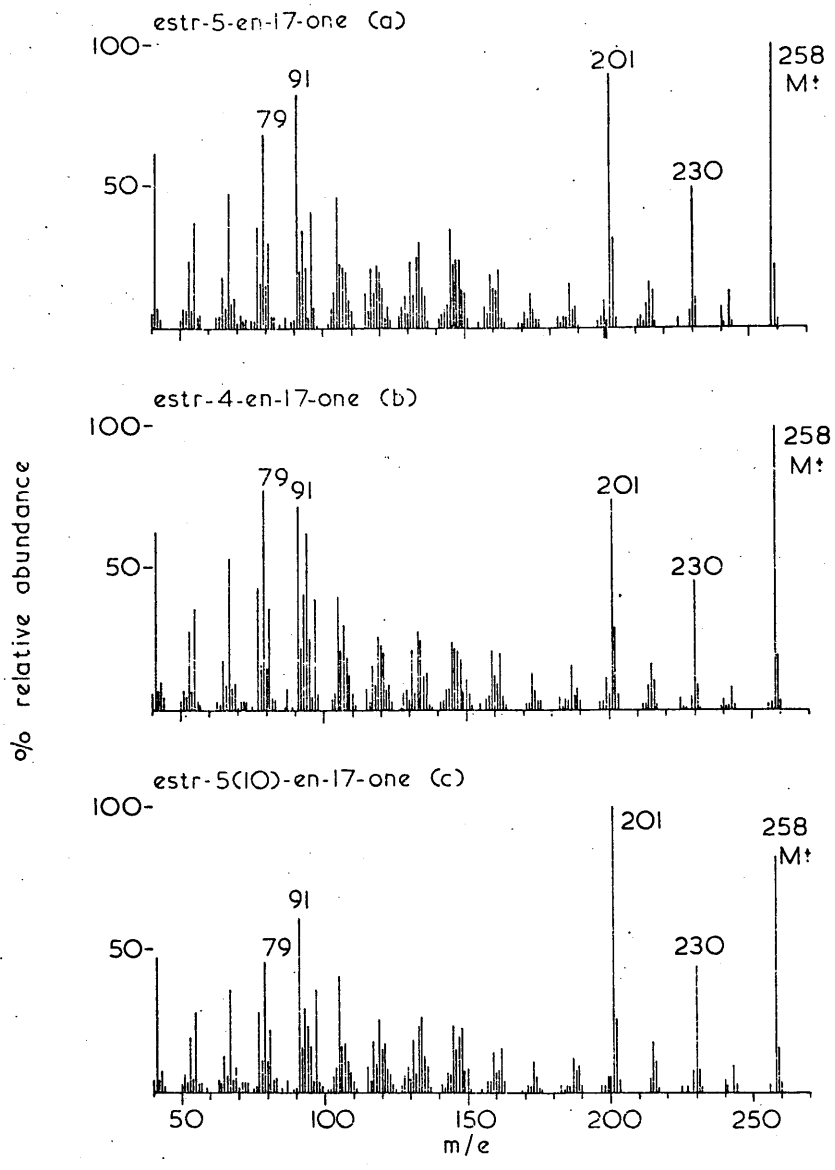
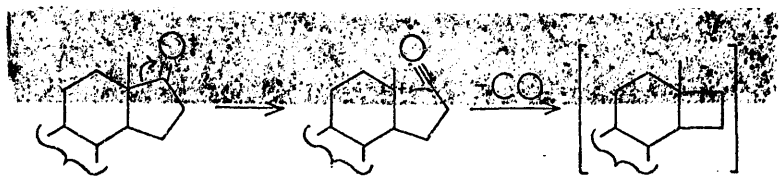


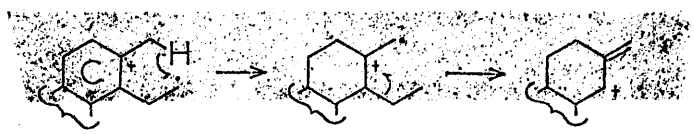
Figure 2.19. Electron impact (EI) mass spectra of the components of the mixture separated in the chromatogram shown in Figure 2.18. Mass spectrometer conditions: LKB 9000, source temperature 270°C, electron energy 70eV.

Figure 2.18. shows the chromatogram of the separation of the three estrenones obtained on an open tubular column installed in the LKB 9000 as compared to a packed column. Mass spectra were obtained without cross-contamination and were consistent with data recorded elsewhere.[255] Notable features in the mass spectra of the resolved isomeric steroids as shown in Figure 2.19 are the prominence of the molecular ion in each case, and their great similarity. The common fragment at m/e 230, $(M-28)^{+\cdot}$, may be the result of the elimination of carbon monoxide from ring D (Scheme 22) or from the loss of C_2H_4. The former is likely as analogous ions appear in the mass spectra of other 17-keto steroids.[255]



Scheme 2.2.

A metastable ion at m/e 276 in all three spectra (not shown in the line diagram) indicates that the fragment ion of m/e 201, which is the base peak from the $\Delta^{5(10)}$ isomer, results at least in part from elimination of C_2H_5 from the ion at m/e 230. This might arise by the process depicted below (Scheme 2.3.)



Scheme 2.3.

The mass spectra contain no ion which could be said to be diagnostic of the structure, possibly because of isomerisation of the double bond sites in the ion source. It is obvious that the specificity of the retention data is critical to the identification of these steroids by g.c.-m.s.

(c) Separation of six C_{19} steroidal alcohols. Approximately 0.5mg of each of the compounds listed in Table 2.5. were weighed into tapered screwcap vials and dissolved in a minimum of dry pyridine and 20µl bis-trimethylsilylacetamide (BSA: Pierce Chemical Company, Rockford, Ill.) and the mixture was heated at

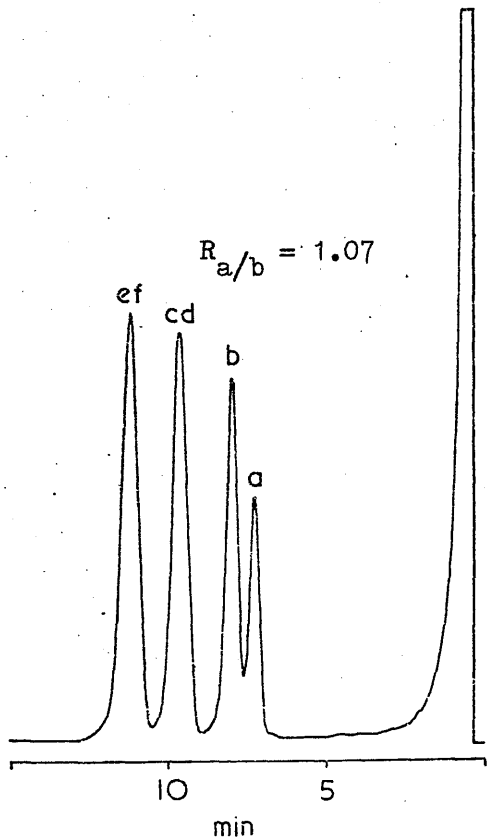


Figure 2.20. Packed column separation of mixture in Figure 2.21.

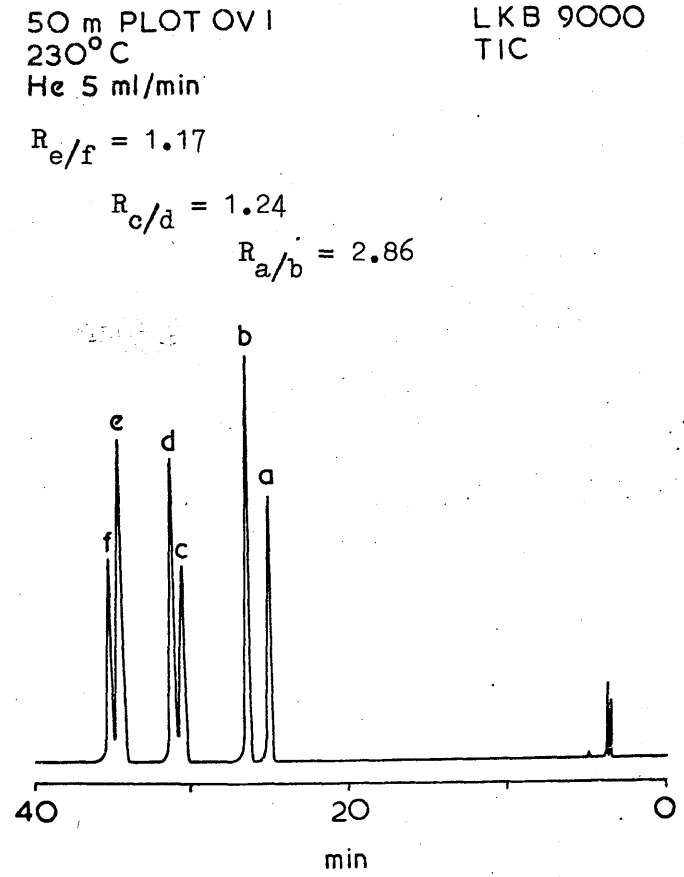


Figure 2.21. TIC chromatogram of separation of 5α-androst-16-en-3α-ol (a), 5β-androst-16-en-3α-ol (b), 5,16-androsta-dien-3β-ol (c), 5α-androst-16-en-3β-ol (d), 5-androsten-3β-ol (e) and 5α-androstan-3β-ol (f) as TMS ethers on 5m Silanox-type (PLOT) OV-1 installed with make-up gas added in LKB 9000.

60°C for 2hours. The solvent and excess reagent were evaporated under a steam of dry nitrogen. The residue was then taken up on sufficient Nanograde hexane to make a concentration of 1μg/μl. 1μl of each of the above solutions was co - injected with n-alkane standards ($n\text{-}C_{20}H_{50}$) on packed column (9ft. 1% OV-1 installed in a Perkin-Elmer Model 881 gas chromatograph at 170°C and on open tubular column (50m Silanox-type glass (PLOT) OV-1 installed in the LKB 9000) at 230°C. Kováts retention indices were calculated and are recorded in Table 2.5.

Table 2.5. Kováts retention index values for six C_{19} steroidal alcohols as TMS ethers.

Diagram reference*	Parent compound	Packed column $I^{170°C}_{OV-1}$	Open tubular column $I^{230°C}_{OV-1}$
(a)	5α-androst-16-en-3α-ol[n]	2168	2223
(b)	5β-androst-16-en-3α-ol[m]	2196	2244
(c)	5,16-androstadien-3β-ol[m]	2234	2290
(d)	5α-androst-16-en-3β-ol[m]	2239	2296
(e)	5-androsten-3β-ol[o]	2273	2330
(f)	5α-androstan-3β-ol[o]	2277	2335

* References to Figure 2.20. and 2.21.
Samples obtained from Ikapharm, Ramat-Gan, Israel, (n), Dr. G.F. Woods, Organon Research Laboratories, Newhouse, Lanarks., (m) and the Medical Research Council Steroid Reference Collection (o).

One microlitre of each of the above solutions was injected on a 10ft. 1% SE-30 packed column installed in the LKB 9000. Reference mass spectra were obtained for each compound at an electron energy of 70eV.

Aliquots of each of the reference solutions of TMS ethers were combined. This mixture was injected on a packed column (9ft. 1% OV-1 installed in a Perkin-Elmer 881) at 170°C and the resulting flame ionization chromatogram is shown in Figure 2.20. The same mixture was chromatographed on a 50m Silanox-type glass (PLOT) OV-1 column installed in LKB 90000. The resulting separation is shown in the TIC chromatogram represented in Figure 2.21. As expected, the 3α-3β hydroxy epimers (a,d) and the 5α-5β epimers (a,b), separable by packed-column chromatography, are well resolved. The Δ^{16},5α-Δ^{5} pair (c and d) is well differentiated from the ring D saturated compounds (e and f).

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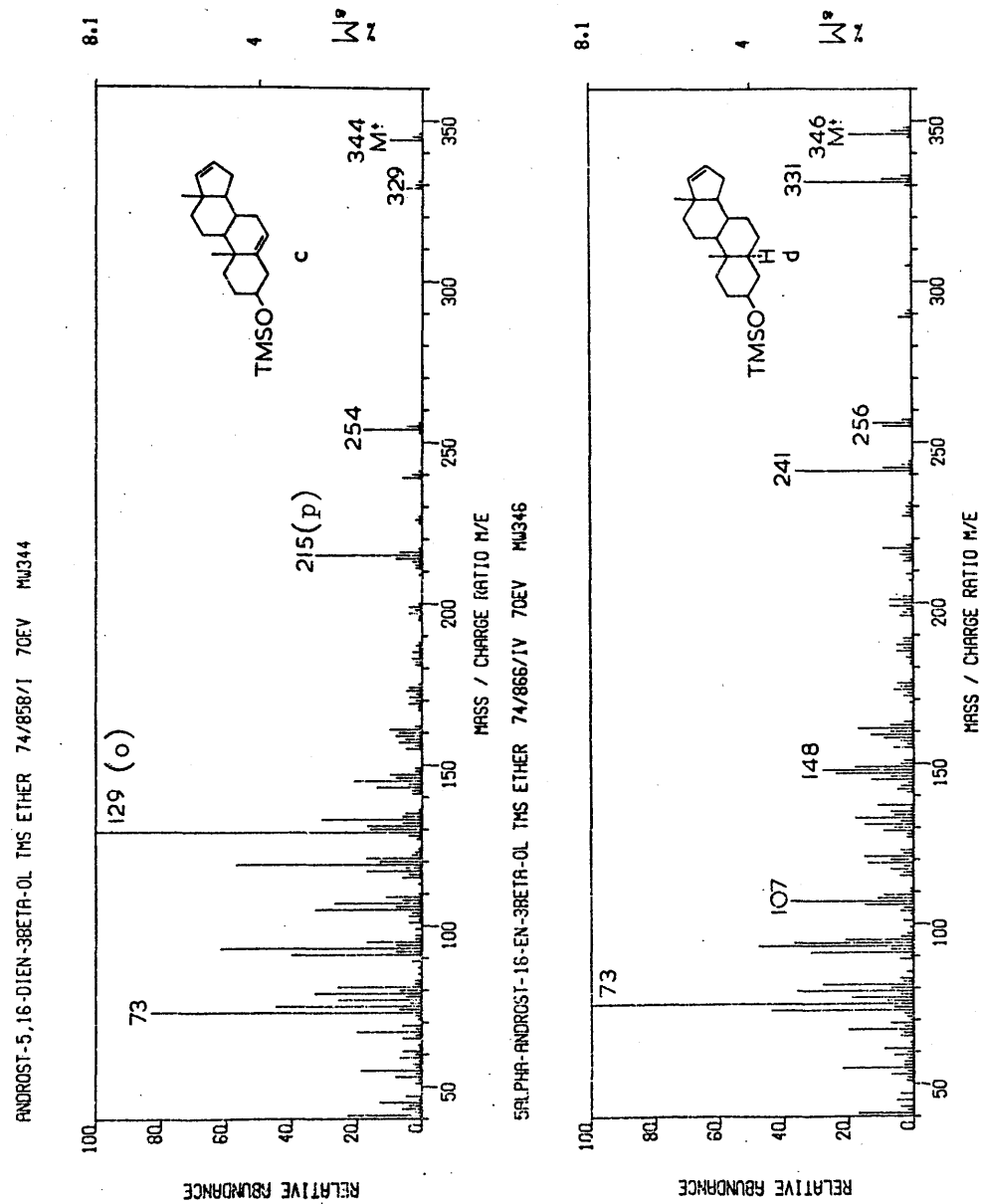

Figure 2.22. Reference mass spectra for 5,16-androstadien-3β-ol TMS ether (c) and 5α-androst-16-en-3β-ol TMS ether (d). Obtained on LKB 9000 electron energy 70eV.

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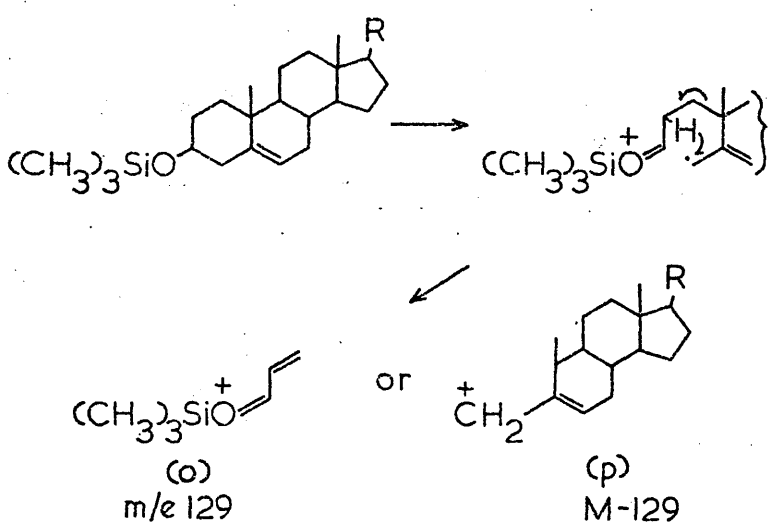

Scheme 2.4. Genesis of ions m/e 129 (o) and M-129 (p) characteristic of TMS ethers of Δ^5-3-hydroxy steroids.[256]

The 3β-trimethylsilyloxy-5α-Δ^5 pairs (c,d and e,f) are resolved by virtue of the improvement in chromatographic resolution afforded by the glass open-tubular column. Mass spectra obtained at the apex of each component peak corresponded closely with those of the individual derivatives. Examples of the mass spectra of a separated 5α-Δ^5 pair are shown in Figure 2.22. for 5,16-androstadien-3β-ol and 5α-androst-16-en-3β-ol as TMS ethers. The mass spectra show the expected similarities, e.g. ions corresponding to $(M-15)^+$, $(M-90)^{+\cdot}$, and $(M-90-15)^+$. The ions of m/e 129 and $(M-129)^+$ are prominent in the $\Delta^{5,16}$ compound, indicating the Δ^5-3-hydroxy TMS ether structure (origin as in Scheme 2.4.). Corresponding ions are of low abundance in the 5α-compound. In the absence of the dominant influence of the Δ^5 double bond, the Δ^{16} double bond enhances ions associated with the loss of the C(19) methyl group, viz. m/e 331 $(M-15)^+$ and m/e 241 $(M-90-15)^+$. Ions of m/e 107 and m/e 148 are analogous to fragments derived from the Δ^{16} hydrocarbon (Scheme 2.1.).

2.4. Evaluation of Gas Chromatography-Mass Spectrometry with Open Tubular Glass Columns: Separation of Steroidal Alkenes

2.4.1. Introduction. Glass open tubular columns offer substantial advantages over packed columns in respect of chromatographic efficiency. Such advances, however dramatic, are unlikely to make it possible to resolve all of the various possibilities for structure variations in organic compounds, particularly with respect to the complexities offered by the large molecules present in biological mixtures. Attention to partial or wholly unresolved peaks in open tubular g.c.-m.s. analyses remain as important as ever.

At the lower chromatographic efficiencies available with packed columns, the g.c.-m.s. analysis of partially or wholly unresolved peaks has been treated in a qualitative[258] and quantitative[257] fashion from a consideration of the full scans obtained at several points during the emergence of the peak from the column. Monitoring structurally specific ions by alternating the accelerating voltage[83] has been developed as a technique for dealing with unresolved components of chromatographic peaks. This idea has been developed by a number of laboratories and instrument manufacturers to extend the selectivity and sensitivity of analysis of chromatographic effluents. This has been recently reviewed by Holmstedt[259,260] and by Gordon.[93] The use of computers allows the

rapid acquisitions of data produced in the course of gas chromatographic-mass spectrometric analyses by continuous repetitive scanning through the course of the entire chromatogram. This field has seen a rapid proliferation of work aimed at the eventual complete automation of the g.c.-m.s. analysis, the reduction of data and its complete interpretation. These trends have been reviewed by Burlingame[261] and more recently by Mellon.[95] The problems associated with this technique have not been completely solved. In particular, overlapping gas chromatographic peaks of isomeric compounds with similar mass spectral properties offer a major stumbling block.[262] Accordingly, more elaborate and useful instrumentation and improved chromatographic efficiencies must operate in concert. Unfortunately foreknowledge of the mass spectra is often prerequisite in the instrumental approaches to unresolved chromatographic peaks. The complexity of natural mixtures makes complete collection of standards prior to analysis difficult or impossible. Investigators must be prepared for the unexpected.

In the following section the separations obtained on open tubular columns are compared with respect to the purity of the mass spectra of the partially resolved components. A series of closely related steroidal alkenes, which were obtained in the course of other work in this laboratory,[255,263] offered this opportunity. The mass spectral consequences of various degrees of resolution of components normally unresolved on packed columns were evaluated by comparison with the mass spectra of the pure compounds. The computative scheme of Appleton and McCormick[257] allowed the quantitative evaluation of the g.c.-m.s. system with open tubular columns. It was intended that these results would serve as a guide for use of the system in the investigation of mixtures of less well defined composition.

2.4.2. Gas chromatography of single components. Table 2.6. shows the Kováts retention index data for two ergostenes and one ergostadiene or a packed and open tubular column. Single compounds were injected with n-alkane standards, n-$C_{28}H_{58}$ and n-$C_{30}H_{62}$, and the retention indices calculated by semi-logarithmic interpolation. The column was a 9ft. 1% OV-1 on 100-120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.) with nitrogen as carrier gas at 40ml/min installed in a Perkin-Elmer Model 881 gas chromatograph operated at 230°C. The retention indices on the open tubular column were obtained by

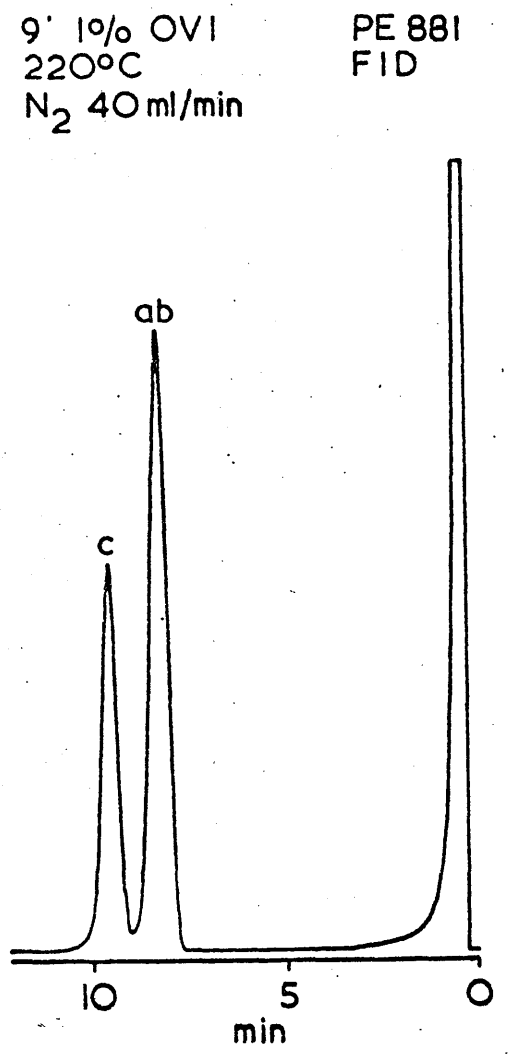

Figure 2.23. Packed column separation of the mixture shown in Figure 2.24.

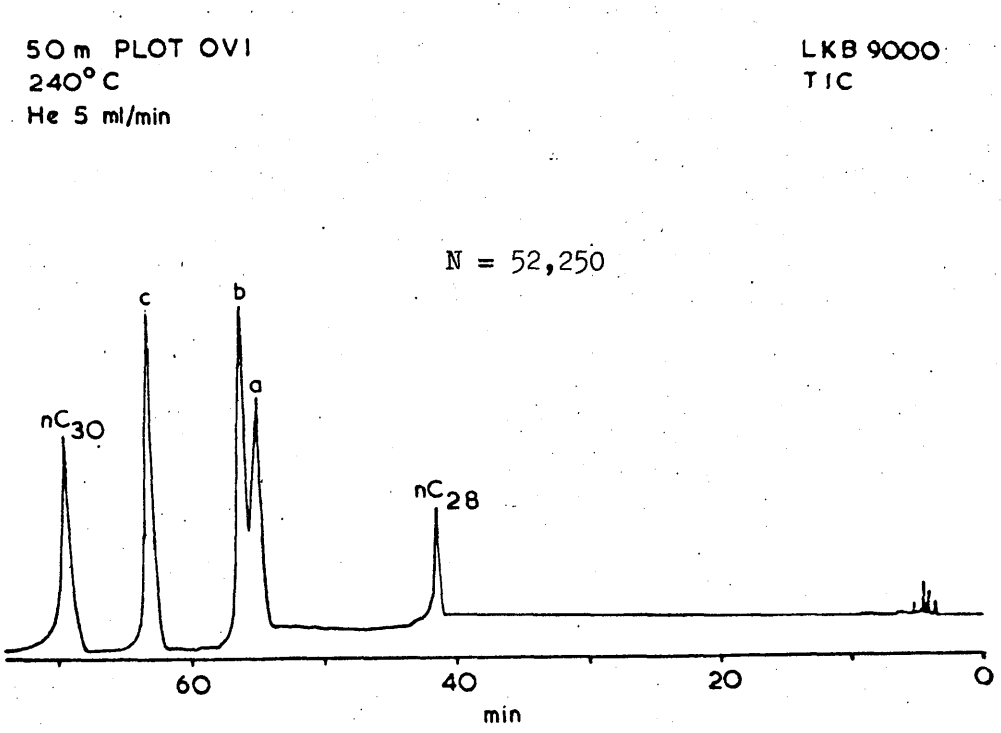

Figure 2.24. Separation on 50m Silanox-type (PLOT) OV-1 installed in LKB 9000 of mixture of 5α-ergosta-7,22-diene (a) 5α-ergost-8(14)-ene (b), and 5α-ergost-7-ene (c). The number of theoretical plates (N) calculated for n-$C_{30}H_{62}$ at column operating conditions.

chromatographing a mixture of all three compounds with n-alkane standards, n-$C_{28}H_{58}$ and n-$C_{30}H_{62}$, and the retention data calculated in the same manner as above. Figure 2.23. and 2.24. show the separations obtained on packed and open tubular columns respectively.

Table 2.6. Kováts retention index values for 5α-ergosta-7,22-diene and two ergostenes on packed and open tubular columns.

Diagram Reference	Compound	Packed column* $I^{OV-1***}_{230^o}$	Open tubular column** $I^{OV-1***}_{250^o}$
a	5α-ergosta-7,22-diene	2860	2920
b	5α-ergost-8(14)-ene	2868	2924
c	5α-ergost-7-ene	2910	2969

* 9ft. 1% OV-1 on 100-120 mesh Gas Chrom Q, N_2 40ml/min.
** 50m x 0.5mm i.d. PLOT OV-1, He 5ml/min.
*** Calculated by semi-logarithmic interpolation with co-injected n-alkane standards, n-$C_{28}H_{58}$ and n-$C_{30}H_{62}$.

Samples obtained by courtesy of Dr. P. Bladon, University of Strathclyde, Glasgow.

The chromatographic data for the cholestenes listed in Table 2.7. required a somewhat different method. The packed column data was obtained by the ordinary way with n-alkane standards, n-$C_{26}H_{54}$ and n-$C_{30}H_{62}$, on a 9ft. 1% OV-1 at 230°C. A slightly different method was adopted for the open tubular column data. The Kováts indices were calculated by a relay method based initially on the index value of 5α-cholestane calculated by semi-logarithmic interpolation of the co-injected n-alkane standards, n-$C_{28}H_{58}$ and n-$C_{30}H_{62}$. The other numbers are obtained by extrapolation from the retention index of this known to the unknown using the slope of the n-alkane.

Table 2.8. correlates the retention data from Tables 2.5. and 2.6. to obtain retention index increments (dI) for the introduction of double bonds into the steroid nucleus. These type of structurally relevant chromatographic transformations will be discussed in detail in Section 3.

A consideration of the retention data recorded in Table 2.7. reveals some ambiguities. The data differ in respect of the values and the implied order of elution of 5-cholestene, 5α-cholest-3-ene

Table 2.7. Kováts retention index values for 5α-cholestane and five cholestenes on packed and open tubular columns.

Diagram reference	Compound	Packed column* $I^{OV-1***}_{230^o}$	Open tubular column** $I^{OV-1****}_{250^o}$
a	4-cholestene	2794	2846
b	5α-cholest-2-ene	2798	2850
c	5-cholestene	2807	2857
d	5α-cholest-3-ene	2800	2861
e	5α-cholestane	2804	2861
f	5α-cholest-8(9)-ene	2816	2871

* 9ft. 1% OV-1 on 100-120 mesh Gas Chrom Q, N_2 40ml/min.
** 50m x 0.5mm i.d. PLOT OV-1, He 5ml/min.
*** Kováts indices calculated by semi-logarithmic interpolation from co-injected n-alkane standards n-$C_{26}H_{54}$ and n-$C_{30}H_{62}$.
**** Kováts indices calculated by a relay method based on 5α-cholestane

$$I^{OV-1}_{250^o} = 2861$$

5α-cholestane obtained from Ikapharm, Ramat-Gan, Israel and cholestenes by courtesy of Dr. P. Bladon, University of Strathclyde, Glasgow.

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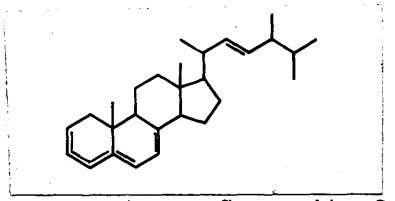
and 5α-cholestane. Rather than an alteration in the order of elution between the two columns it is considered that this reflects the difficulty in producing consistent retention data for closely related compounds. Comparison of data obtained from separate chromatograms for compounds within a narrow retention index range is difficult. This is accentuated where there is a wide gap between internal standards, i.e., even numbered n-alkanes, and where limited quantities of material make replicate determinations and statistical treatment impractical. These problems arise for isothermal and temperature programmed modes in both packed and open tubular column gas chromatography. While other laboratories have quoted very high standards for precision and accuracy,[264] these can be attained only where column temperature and carrier gas flow rate carefully controlled within extremely narrow limits. The isobaric operation of the open tubular columns connected to the dry injection system is not subject to the compensatory control of mass flow controllers in common use with liquid injection in packed column gas chromatographs. This renders column temperature control even more critical for open tubular operation. Improved chromatographic resolution permits more self-consistent data in respect of order of elution and the use of a relay system with a

Table 2.8. Retention index increments (dI) for double bonds in steroidal hydrocarbons on methyl silicone stationary phase (OV-1)*

Double bond	Steranes compared	Carbon atoms	I
Δ^4	Δ^4/5α-sterane	$C_{27}(C_8)$	-15
Δ^2	Δ^2/5α-sterane	$C_{27}(C_8)$	-11
Δ^5	Δ^5/5α-sterane	$C_{27}(C_8)$	-4
Δ^3	Δ^3/5α-sterane	$C_{27}(C_8)$	0
$\Delta^{8(9)}$	$\Delta^{8(9)}$/5α-sterane	$C_{27}(C_8)$	+10
Δ^{22}	$\Delta^{7,22}/\Delta^7$	$C_{28}(C_9)$	-49

* Based on data recorded in Tables 2.6. and 2.7. g.c. conditions: 50m Silanox OV-1, He 5ml/min, 250°C.



Composite formula

Correlation Chart

Nucleus/Sidechain

	A	B	C	D	E	F	G	H
1	2850	2861	2846	2857	2871	-	-	2861
2	-	-	-	-	-	2969	2924	-
3	-	-	-	-	-	2920	-	-

Nucleus

A	C_{19}-5α-Δ^2
B	C_{19}-5α-Δ^3
C	C_{19}-Δ^4
D	C_{19}-Δ^5
E	C_{19}-5α-$\Delta^{8(9)}$
F	C_{19}-5α-Δ^7
G	C_{19}-5α-$\Delta^{8(14)}$
H	C_{19}-5α

Sidechain

1	C_8
2	C_9-24-methyl
3	C_9-24-methyl-Δ^{22}

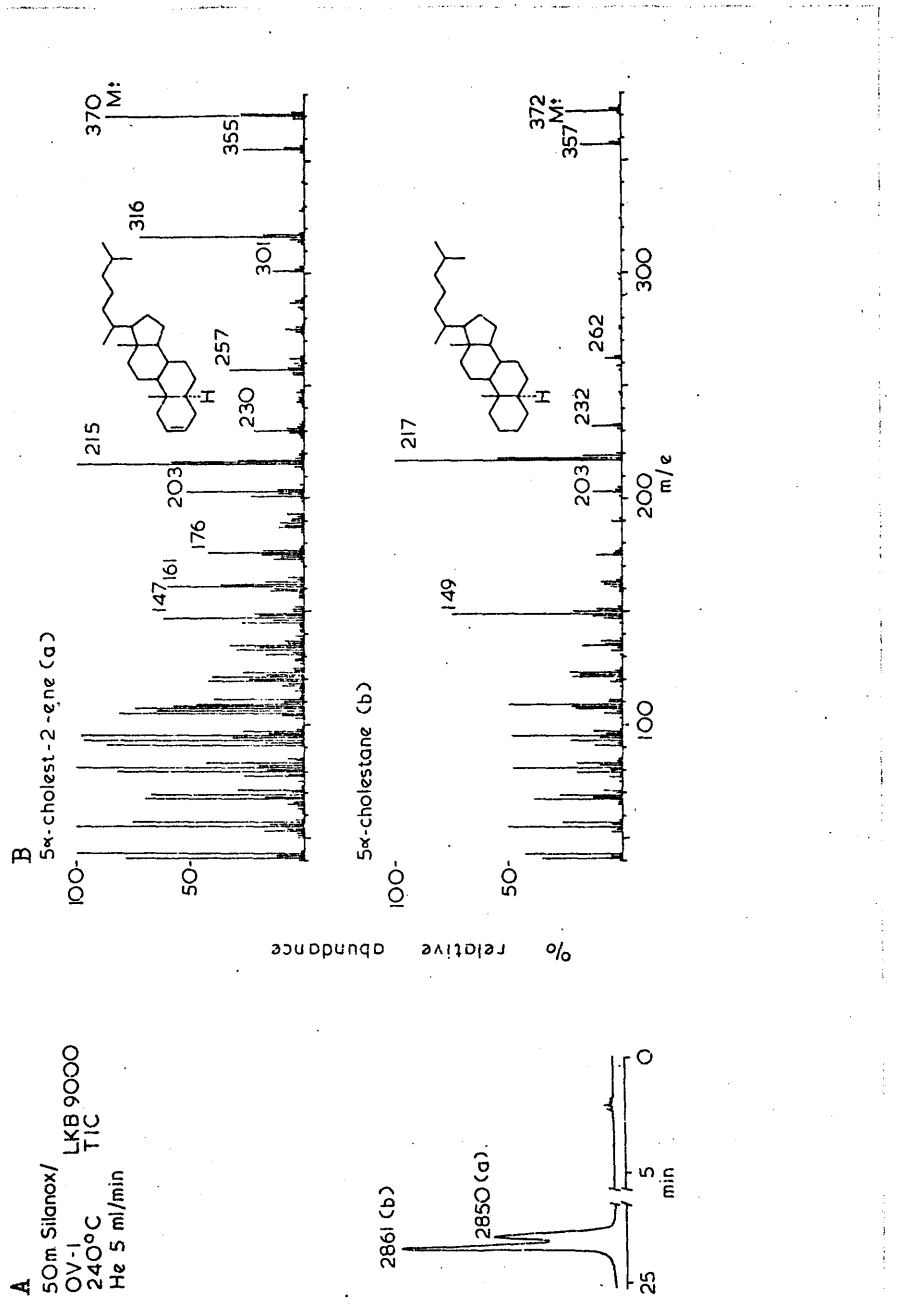


Figure 2.25. Total ionisation current (TIC) chromatogram (left) and electron impact (EI) mass spectra (right) of the analysis of a mixture of 5α-cholest-2-ene (a) and 5α-cholestane (b). Retention indices appear on the chromatogram.

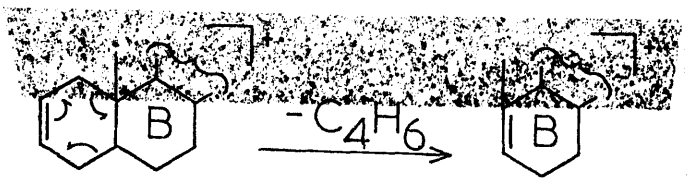
close internal standard improves precision. However, in our experience, it is a very difficult task to construct an accurate body of isothermal retention data for compounds differing from one another by 10 Kováts units or less with either packed or open tubular columns. The precision and accuracy in temperature programmed operation is somewhat better and this is presented in the portion of this thesis dealing with the application of these columns to urinary steroid profiles (Section 4.3.2).

The total ionization current chromatogram in Figure 2.25. shows a phenomenon common with the use of open tubular columns on the LKB 9000, the change in baseline from a previously very stable condition as the peaks enter the mass spectrometer. It is considered that this reflects a change in the ionization characteristics of the source. This baseline shift decreases or disappears with successive injections. A change in column flow rate might also account for this, however the lack of improvement in the quality of retention data when this phenomenon disappears or with normal chromatograph operation offers indirect evidence against this possibility.

2.4.3. Mass spectrometric evaluation of the resolution of 5α-cholest-2-ene and 5α-cholestane. Figures 2.25A shows the resolution obtained of a mixture of 5α-cholest-2-ene and 5α-cholestane on a 50m x 0.5mm i.d. Silanox-type glass (PLOT) OV-1 column installed in the normal manner in the LKB 9000, with column temperature at 240°C and helium carrier gas at 5ml/min. Standard mass spectra of the six steroidal hydrocarbons listed in Table 2.8. as single components were obtained in the same g.c.-m.s. system with electron energy 70eV. Figure 2.25B shows the standard mass spectra obtained for 5α-cholest-2-ene and 5α-cholestane. Other reference spectra appear in Appendix B.

Here the chromatographic separation is relatively difficult but the mass spectra are highly distinctive. Cleavage through ring D yields ions at m/e 217 and 215 respectively, and the nuclear double bond position at C(2) is well characterised by the ion at m/e 316 resulting from retro-Diels-Alder elimination of C_4H_6 (Scheme 2.5.).

The mixture of 5α-cholest-2-ene and 5α-cholestane was chromatographed on the open tubular g.c.-m.s. system and scans were obtained at the summit of each peak. Because of the sharpness of the peaks this required initiating the scan somewhat before the true peak



m/e 370 m/e 316

Scheme 2.5.

maximum and allowing the mass spectrometer to scan through the top of the peak. The two scans obtained were mixtures of the two components, proportions of which were determined by the resolution effected by the column and by any remixing which may have occurred in the g.c.-m.s. interface. Further re-mixing may occur in the ion source, unless the pumping speed is adequate to ensure that the residence times of the samples are short compared with the differences in retention times.

The elucidation of the composition of the mixed spectra obtained was accomplished according to the method of Appleton and McCormick.[257] It has been shown that hydrocarbon isomers on electron bombardment in the mass spectrometer produce the same total ionization per mole of sample.[265] Assuming this to be true for steroidal hydrocarbons the analysis of the two component mixture proceeds as follows:

Consider the mass spectra of 5α-cholest-2-ene and 5α-cholestane (Figure 2.25). Suitable peaks for the quantitative analysis of a mixture of these compounds[257] occur at m/e 215 and m/e 217. m/e 215 is 3.02% of the total ionization ($\Sigma_{40}I$) for 5α-cholest-2-ene and 0.08% for 5α-cholestane. This means that in the mass spectrum of a mixture of these two compounds the peak height will be

$$H_{215} = 3.02C_{(e)} + 0.08C_{(b)}. \qquad \text{(eq. 2.7.)}$$

Where H_{215} is the mixture peak height at m/e 215 in arbitrary units and $C_{(e)}$ and $C_{(b)}$ are the relative molar concentrations of 5α-cholest-2-ene and 5α-cholestane respectively. Similarly for the peak height at m/e 217.

$$H_{217} = 0.85C_{(e)} + 8.21C_{(b)} \qquad \text{(eq. 2.8.)}$$

These two relations form two equations in two unknowns. A graphic technique is the most convenient means of obtaining the simultaneous

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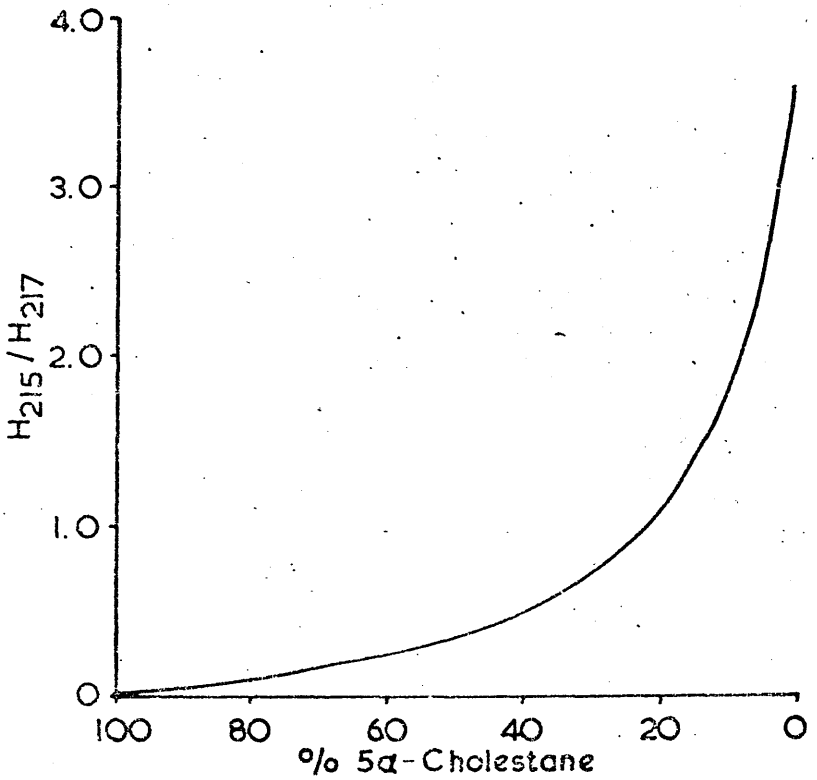

Figure 2.26. Calculated ratio of peak heights at m/e 215 and m/e 217 (H_{215}/H_{217}) versus composition for mixtures of 5α-cholestane (e) and 5α-cholest-2-ene (b).

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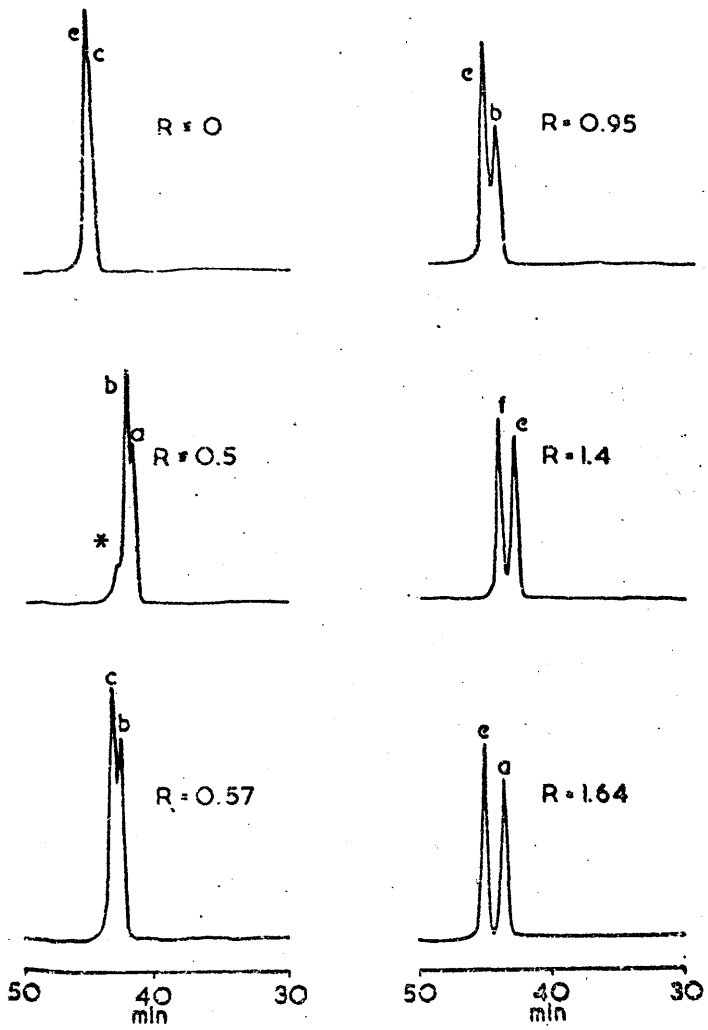

Figure 2.27. Separations of six selected two components synthetic mixtures of 4-cholestene (a), 5α-cholest-2-ene*(b), 5-cholestene (c), 5α-cholestane (e) and 5α-cholest-8(9)-ene (f) obtained on 50m Silanox-type (PLOT) OV-1 column installed in LKB 9000. Mass spectra at 70eV electron energy, multiplier sensitivity 190 (2.3kV), obtained at the apex of each peak resolved chromatographically

* 5α-cholest-3-ene present as contaminant in sample of 5α-cholest-2-ene.

solutions to these equations. A range of possible solution compositions are chosen and the ratios of peak heights at m/e 215 and m/e 217 are calculated. These are plotted (Figure 2.26) and the composition of any mixed spectrum determined for any ratio of peak heights obtained. The results of this procedure are summarized in Table 2.9.

Table 2.9. Mass spectral evaluation of the separation of 5α-cholest-2-ene and 5α-cholestane.*

Peak	H_{215} mm	H_{217} mm	H_{215}/H_{217}	Composition
b	11.5	3.3	3.48	2% 5α-cholestane
e	0.4	5.4	0.07	86% 5α-cholestane

* See Figure 2.25.

2.4.4. Correlation of chromatographic resolution with mass spectral purity. The array of compounds represented in Table 2.7. offered the opportunity of assessing the chromatographic resolution obtained on the open tubular g.c.-m.s. system in terms of the quality of the mass spectral data. The procedure outlined above was applied to six two-component mixtures selected to afford resolutions varying from substantially unresolved to baseline separation. These are shown in Figure 2.27. Ions specific to the compounds in each pair were selected and the calculations performed. Table 2.10. summarizes the results obtained for the six mixtures.

Figure 2.28. represents these results graphically. The data appear as a graph of peak composition for the first and second peak of the pair versus the resolution (R) between the two peaks. These compositions are in terms of the mole percentage of the first component in each of the peaks. From this representation it may be determined that in the case of the first component the mass spectrum obtained at the top of the peak is contaminated by the second component down to a resolution of about R = 0.8. However, for the second peak a complete resolution, R = 1.5, is necessary to obtain mass spectral data free from the influence of the first eluted peak. This asymmetric behaviour is due to a combination of incomplete chromatographic resolution, peak tailing in the chromatographic system, mixing in the g.c.-m.s. interface, and

Table 2.10. Summary of results of the calculations of mass spectral composition of peak resolved in the six two-component mixtures.

1st Peak/2nd Peak	Resolution	Kováts index difference	Composition 1st Peak	Composition 2nd Peak	Peak ratio in calculation
5-cholestene/ 5α-cholestane	0	4	single peak:	52% 5α-cholestane	H_{215}/H_{217}
4-cholestene/ 5α-cholest-2-ene	0.5	4	91% 4-cholestene	13% 4-cholestene	H_{108}/H_{215}
5α-cholest-2-ene/ 5-cholestene	0.57	7	100% 5α-cholest-2-ene	19% 5α-cholest-2-ene	H_{201}/H_{203}
5α-cholest-2-ene/ 5α-cholestane	0.95	6	98% 5α-cholest-2-ene	14% 5α-cholest-2-ene	H_{215}/H_{217}
5α-cholestane/ 5α-cholest-8(9)-ene	1.4	10	100% 5α-cholestane	7% 5α-cholestane	"
4-cholestene/ 5α-cholestane	1.64	15	100% 4-cholestene	6% 4-cholestene	"

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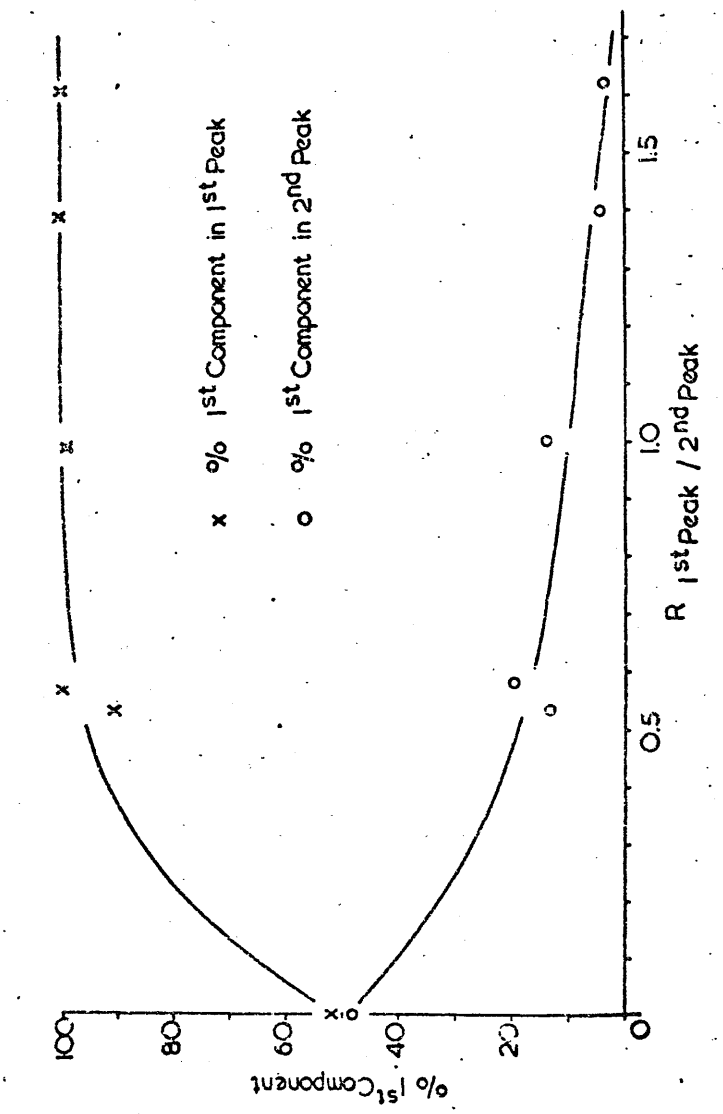

Figure 2.28. Mass spectral composition of peaks of components of two-component mixtures versus the resolution (R) between the two peaks obtained on LKB 9000 with 50m Silanox-type (PLOT) OV-1. Composition is expressed as the percentage of the first eluted component in the mass spectra at the apex of the peak of each component resolved chromatographically. Data obtained from the mixtures shown in Figure 2.27.

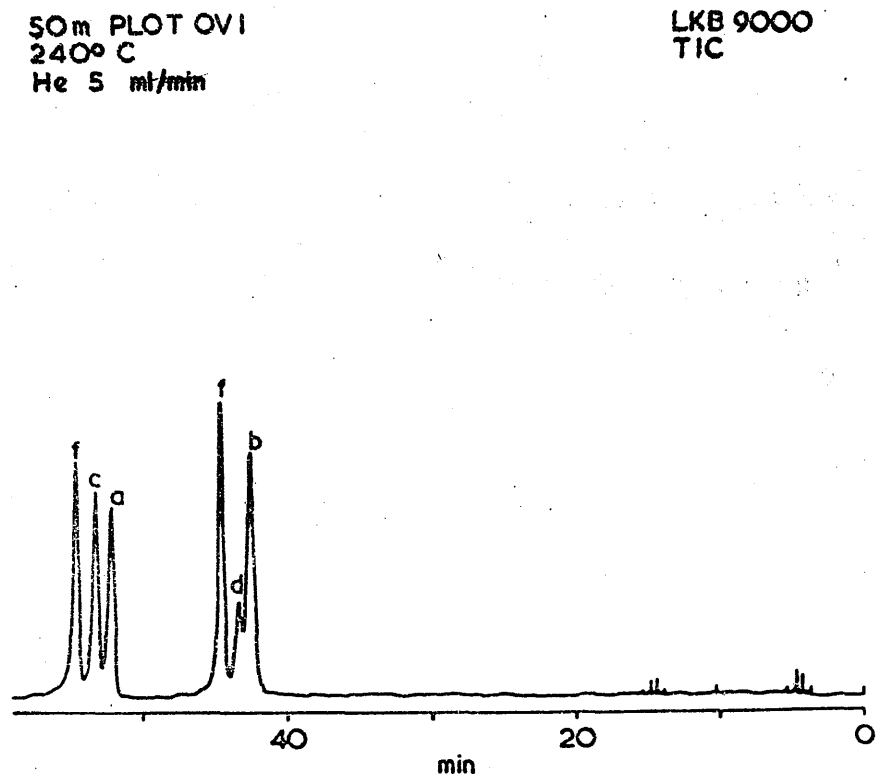

Figure 2.29. Separation of a mixture of 5α-cholest-2-ene (b), 5α-cholest-3-ene (d) and 5α-cholest-8(9)-ene (f) and a mixture of 4-cholestene (a) 5-cholestene (c) and 5α-cholest-8(9)-ene (f). Successive dry injections at a 10min interval on 50m Silanox-type (PLOT) OV-1 installed in LKB 9000 with helium flow rate at 5ml/min.

mixing in the source arising from the unpumped residue of the first peak present when the second peak is scanned. The extent to which each of these factors participate is inaccessible with the instrument in the present configuration, however the overall effect is of significance in the evaluation of data obtained from mixtures of unknown composition. It has been observed previously that a resolution of R = 1 is generally necessary for unbiased mass spectra of mixtures by g.c.-m.s.[233] It seems that for wholly uncontaminated mass spectra a more rigorous standard may be necessary for later eluting peaks in closely-grouped multiplets such as those represented in Figure 2.29.

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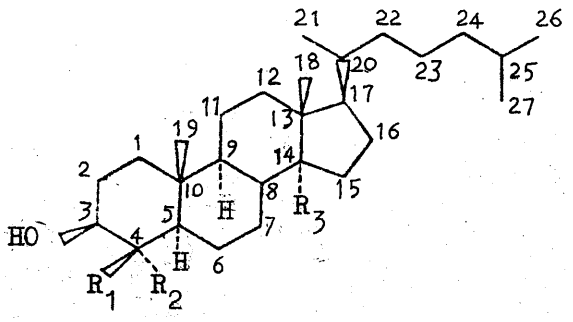
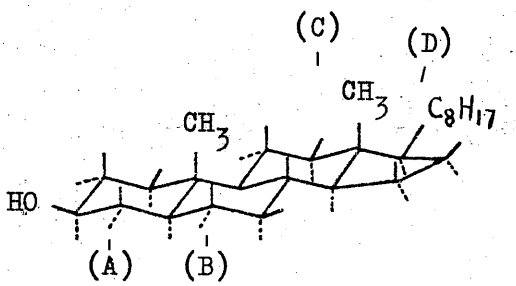


(a)



(b)

Figure 3.1. (a) The general structure and accepted numbering system for sterols, (b) stereochemistry of the typical sterol, 5α-cholestan-3β-ol. (Conventional designation of the rings of the nucleus is shown in brackets.)

3. APPLICATION OF GLASS OPEN TUBULAR CHROMATOGRAPHIC COLUMNS TO THE ANALYSIS OF STEROL MIXTURES BY COMBINED GAS CHROMATOGRAPHY-MASS SPECTROMETRY.

3.1. Introduction

3.1.1. Structure, stereochemistry and nomenclature of sterols.

The structure of the major animal sterol, cholesterol, was elucidated in 1932 after detailed investigations which culminated in proposals by Wieland[266] and Rosenheim and King.[267] (A detailed account of the inquiry is given by Fieser and Fieser[268].) It was found to consist of a cyclopentanoperhydrophenanthrene skeleton with an eight carbon atom side chain attached at position 17, and two angular methyl groups at positions 10 and 13. The structure is completed by a secondary hydroxyl group at carbon 3 and a double bond between carbon atoms 5 and 6. Sterols are a class of compounds related to this basic structure and may differ by extra substituents at 4, 14 and 24, moreover, the 5,6 double bond may be saturated and/or other double bonds present. Figure 3.1a. shows this general structure with the accepted numbering scheme as proposed by the I.U.P.A.C.[269]

The saturated analogue of cholesterol is 5α-cholestan-3β-ol whose stereochemistry is shown in Figure 3.1b. This and sterols in general consist of three fused cyclohexane rings (A, B, and C) plus a terminal cyclopentane ring (D). The low energy conformation of the rings A, B, and C is all-"chair" (Figure 3.1.).[268,270] In this arrangement group interactions are minimized. This stereochemical concept is essential for understanding and predicting the course of chemical reactions involving these compounds. Most naturally occurring sterols are of the same configuration as 5α-cholestan-3β-ol. Rings A, B, C and D are fused in the trans configuration. Atoms or substituents at positions 8,9,10,13 and 14 are 8β,9α,10β,13β, and 14α, respectively (Figure 3.1a, α: projecting below the plane of the paper and β: projecting upwards). Some steroids contain a cis A/B ring fusion (e.g. coprostanol, 5β-cholestan-3β-ol) and so the stereochemistry of this ring junction is defined in the nomenclature (5α-: trans A/B, 5β-: cis A/B). The 18 and 19 angular methyls and the side-chain at carbon 17 are in the β configuration.

C(20) in the side chain is chiral and most naturally occurring sterols are of the 20-R configuration.[271] The hydrogens at carbon 24 are pro-chiral so that when substituted by an alkyl group two epimers are possible. Under the Plattner convention as extended by Fieser these are designated α or β. I.U.P.A.C. convention requires

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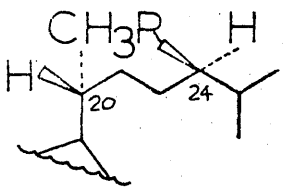
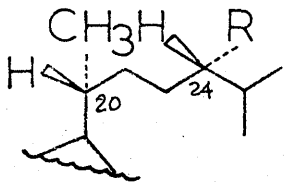
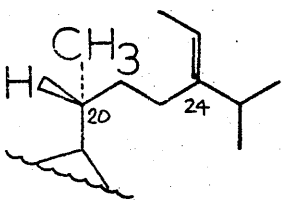


20β,24α
or
20R,24R

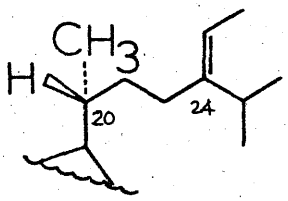
(a)



20β,24β
or
20R,24S



20β,24E
or
20R,24E



20β,24Z
or
20R,24Z

Figure 3.2. Stereochemistry of the side-chain of (a) 24-alkyl and (b) 24-alkylidene sterols.

C(24) configuration be specified as R or S as defined by the revised sequence rule (Figure 3.2.).[271,272]

A useful shorthand notation in referring to sterol structures is to specify the number of carbon atoms and the position of the double bonds. Thus, for demethyl sterols (i.e. sterols lacking extra methyl substitution at carbons 4 or 14), a C_{29}: $\Delta^{5,7,22}$ sterol implies 24-ethylcholesta-5, 7, 22-trien-3β-ol without specification of the configuration at carbon 24.

3.1.2. Function and distribution of sterols. Sterols have proved virtually ubiquitous in nature and have been the subject of very extensive study. A number of reviews have appeared covering the sterol constituents of groups of organisms broadly covering the phylogenetic spectrum. Some of these appear in Table 3.1. Vertebrates are omitted from this list, as steroid content in these is complicated by the presence of other components associated with diet and microbial modification. Sterols are found in most eukaryotic organisms and recent investigations have detected unsaturated sterols in prokaryotic bacteria and blue-green algae at relatively low levels. In most higher plants the major sterol is β-sitosterol. Lower animals, protozoa and some forms of plant life contain a great variety of sterols. Most plant sterols possess Δ^5 or less commonly Δ^7 components with, in some cases, an extra position of unsaturation at C(22). In contrast to "animal sterols" plant sterols are commonly alkylated at C(24) and the stereochemistry of this position has been shown to be of taxonomic significance.[273] Higher plants generally contain sterols possessing the 24α configuration such as campesterol (C_{28}: Δ^5: 24R-methyl); β-sitosterol (C_{29}: Δ^5: 24R-ethyl). Brassicasterol (C_{28}: $\Delta^{5,22}$: 24R-methyl) and Δ^{25} sterols are also known and possess a 24β configuration.[274] On the other hand, algae and fungi produce sterols exclusively in the 24β stereochemistry; such as, ergosterol (C_{28}: $\Delta^{5,7,22}$: 24R-methyl), poriferasterol (C_{29}: $\Delta^{5,22}$: 24R-ethyl) and clionasterol (C_{28}: Δ^5 : 24S- ethyl).[275,276]

24-Ethylidene sterols also occur with stereochemistries specific to the organism. Fucosterol (C_{29}: $\Delta^{5,E\text{-}24(28)}$) with the 24E configuration is found in brown algae[275] and some fungi[277] while the 24Z isomer, Δ^5-avenasterol (C_{29}: $\Delta^{5,Z\text{-}24(28)}$) occurs in higher plants[278,279] and green algae.[275,276] Figure 3.2. illustrates the stereochemistry of the unsubstituted, 24-alkyl and 24-alkylidene sterols.

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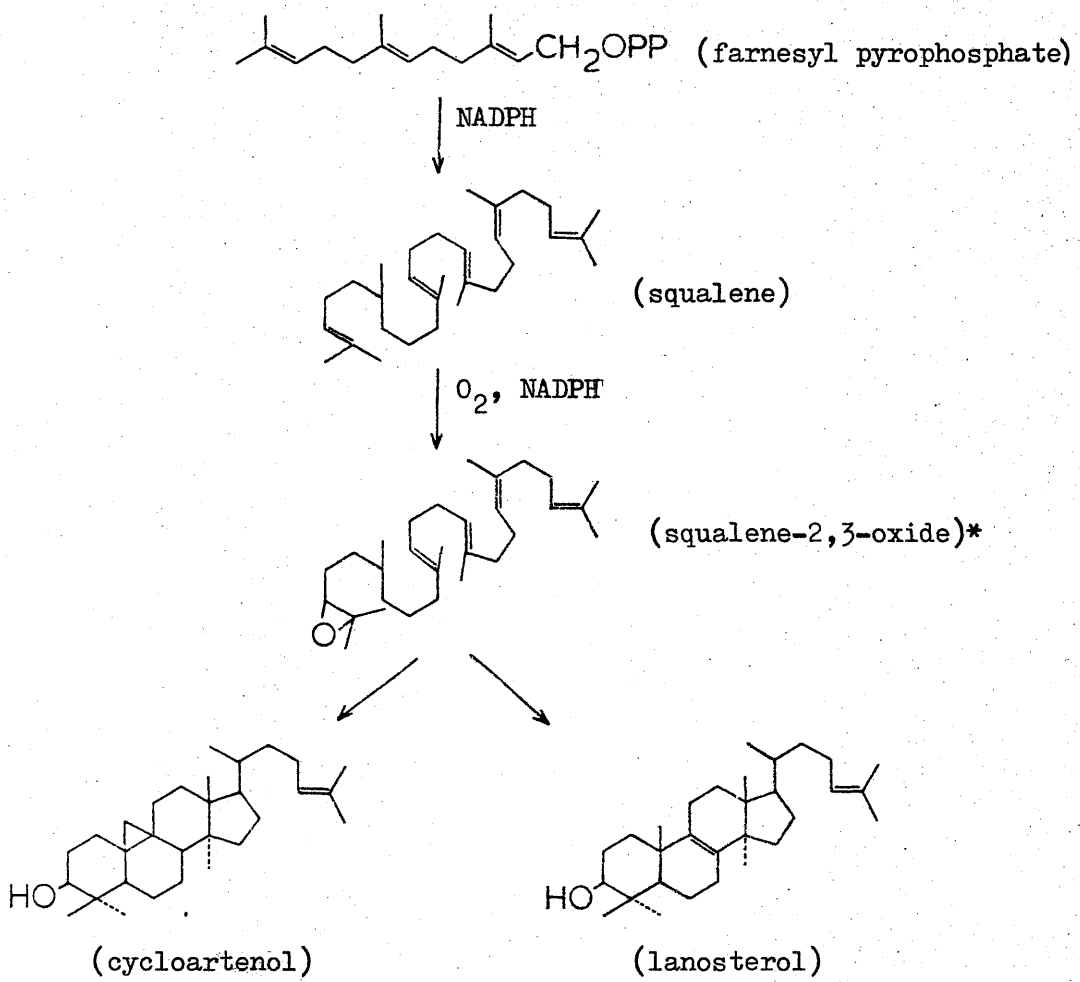

Figure 3.3. Part of a general scheme for sterol biosynthesis.

(* chair-boat-chair-boat conformation)

Compounds with structures retaining the steroid carbon skeleton have been frequently identified in petroleum and in ancient and modern sediments.[142] The presence of these compounds in geological materials ranging in age from contemporary to Precambrian is consistent with their being derived from biogenic sterols and related compounds.[280] The origin and fate of sterols and other carbon compounds in contemporary sediments is one object of the burgeoning field of environmental organic chemistry and these investigations have important parallels in the fate of similar materials in older geological formations. Some leading references the occurrence of sterols in biological and paleobiological materials are briefly summarized in Table 3.1.

In vertebrate animals cholesterol functions as precursor for other steroids, steroid hormones and bile acids and as a membrane component.[297] In eukaryotic organisms the main role of steroids is probably as membrane component.[298] In insects, cholesterol and other sterols obtained in the diet are utilized to synthesize ecdysterone, a moulting and metamorphosis hormone.[299] In higher plants, in addition to the role in membrane structure, cholesterol and β-sitosterol may play a hormonal role.[300,301] The presence of such a wide variety of structurs of sterols in various organisms, and especially in plants, remains an intriguing problem.

3.1.3. Summary of sterol biosynthesis. Some organisms are not capable of synthesizing sterols. These materials are, in these cases, generally dietary requirements. In all organisms, plants and animals, where sterol biosynthesis takes place, the general scheme is similar.[302,303] This might indicate that the system was established early in the evolutionary development of biological systems. Figure 3.3. shows part of this general scheme for sterol biosynthesis.[302,304,305] In this figure the first noted intermediate is farnesyl pyrophosphate which is produced *via* isoprenoid condensations involving isopentenyl and dimethylallyl pyrophosphate. Through squalene and the concerted cyclization of the chair-boat-chair-boat conformation of squalene-2,3-oxide, the first cyclic intermediate in sterol biosynthesis is produced. In higher plants[304] and algae[276] this is cycloartenol, and in animals[302] and fungi[287] it is lanosterol. This specificity may be an aspect of the cyclisation mechanism since certain plants may also utilize lanosterol in phytosterol biosynthesis.[303,304]

Table 3.1. Occurrence of sterols in biological and paleobiological materials.

Category	Main subgroups	Reference
Monera*	bacteria	281,282,283
	blue-green-algae	284,285
Protista*	algae	275,276,286
	fungi	276,277,287
	protozoa	276
	slime moulds	288,289
Metaphyta*	bryophytes	273,274,290
	tracheophytes	
Metazoa*	sponges	291
	coelenterates	292
	worms	286
	molluscs	293
	arthropods	294
	echinoderms	295
Recent sediments		296
Ancient sediments		142

* Phylogenetic class of the living system.

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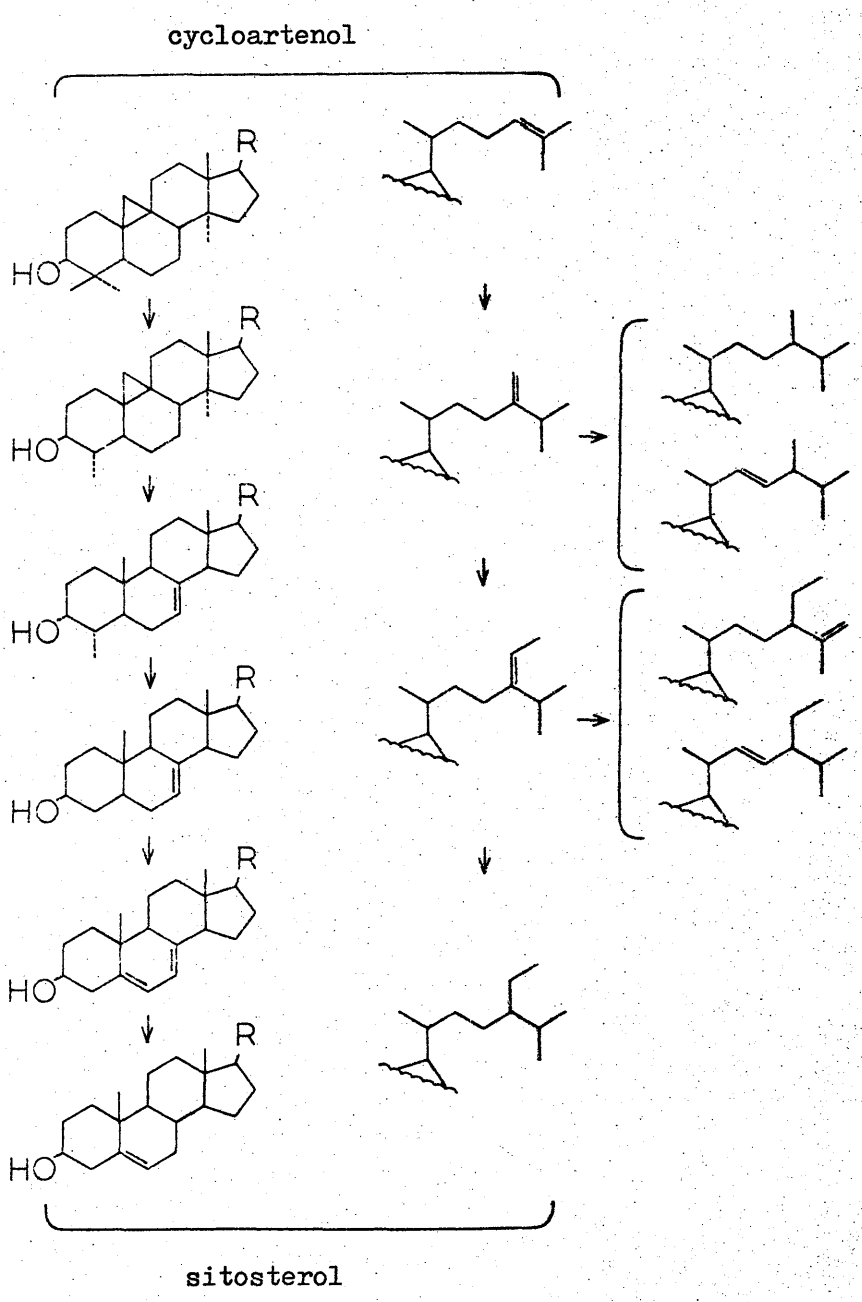

Figure 3.4. The two sets of transformations involved in the conversion of cycloartenol into sitosterol in plants.[273]

In animals the further metabolism of lanosterol to cholesterol involves a number of modifications which may be summarized as:

(a) Sequential oxidative removal of the 4,4-gem-dimethyl and the 14α-methyl group.

(b) Isomerization of the 8,9 double bond to the 5,6 position by a sequence of $\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7} \rightarrow \Delta^5$ transformations.

(c) Reduction of the 24,25 double bond.

These processes are remarkably nonspecific in their order and in animals, where sterols also constitute a part of the diet and as the products of microbial activity, the diversity of sterols and related steroidal compounds is formidable.

Generally, the abundant sterol constituents of plants are alkylated at carbon 24. The transformations involving the polycyclic nucleus and the sidechain in plants are summarized in Figure 3.4.[273] Modification of nucleus and sidechain may be separately considered as correspondence varies among species.[306] Alkylation has been shown to proceed via 24-methylidene intermediates. Further alkylation has proved to be very complex in its mechanism[307] and is the subject of current interest in several laboratories.

3.1.4. Techniques for the analysis of complex sterol mixtures. Difficulties in the isolation and characterisation of complex mixtures of sterols have led to the development of elaborate chromatographic procedures.[308] The most successful investigations involve the complementary use of several chromatographic techniques (for example, 295 and 309). On occasion advances in chromatographic techniques have demonstrated several components where originally only a single "new sterol" had been identified (for example 292 and references therein). As has been discussed earlier, g.c. and g.c.-m.s. are particularly well suited for the analysis of complex mixtures. However, preliminary procedures incorporating other separation techniques may be desirable or necessary. Prior separation may lead to the enrichment of minor components which might be difficult or impossible to characterize in the original mixture. Preliminary chromatographic procedures may also provide complementary information by providing structural group separations. These manipulations are perhaps most significant where g.c. and g.c.-m.s. are ambiguous for the structure in question. Chemical or preparative chromatographic operations may be essential for the isolation of individual components for subsequent analysis by

chemical or other instrumental means (typically, n.m.r., o.r.d., c.d., u.v., i.r.).

Table 3.2. summarises analytical and preparative separation methods, other than gas chromatography, which have found application in the analysis of sterol mixtures. A number of methods used for the chemical modification of sterols or derivatization and subsequent separation are also included. A recent paper by Djerassi and co-workers[310] provides an up to date survey of methods for the extraction and fractionation of complex natural sterol mixtures.

3.1.5. Introduction to gas chromatography and combined gas chromatography-mass spectrometry of sterols. Gas chromatography has been employed in the analysis of sterols as free compounds[341,342] or derivatized as acetates,[343] as halogen substituted esters,[344] methyl ethers,[345] trimethylsilyl ethers,[346] and as halogen substituted silyl ethers.[347] Derivatisation is employed to improve the chromatographic behaviour of sterols and improve detectability with selective detectors. A variety of stationary phases have been employed in the separation of sterol mixtures using packed column gas chromatography.[348,349,350] Polar phases may offer useful selectivity of analysis, the separation of cholesterol and cholestanol on CHDMS-PVP,[343] QF-1 and F-60[351] being particularly noteworthy. These and other aspects of the analysis of sterols by packed column gas chromatography are discussed in a useful review by Knights.[351]

While the analysis of sterol mixtures on two or more phases may be informative, in the analysis of complex mixtures columns of higher resolving power are particularly important. Long packed columns of conventional internal diameters (approximately 3.5mm)[352] and narrow bore packed columns (less than 2mm)[246] have been utilized. Metal open tubular columns have been employed, but may be somewhat handicapped by structure dependent absorption.[353] Glass open tubular columns have been demonstrated to be superior for such applications[185,354,355] and these are now finding wider application.

Systematic efforts to correlate steroid structure with gas chromatographic retention behaviour generally involved extension of the principles proposed by Bush[356] for partition chromatography. To reiterate the relation discussed earlier (Section 1.2.4.), the retention time (r) of a sterol may be expressed as

$$r = r_n \times k_a \times k_b \times \ldots\ldots\ldots \qquad \text{(eq. 3.1.)}$$

Table 3.2. Separation techniques (other than gas chromatography) used in sterol analysis.

Separation Criteria	Method	Comments	References
Group separation according to methyl substitution at C-4	Alumina column chromatography	Sepn. of 4,4-dimethyl/4-methyl 4-demethyl sterols	309,311
	Alumina thin layer chromatography	"	311,312
Group separation according to alkyl substitution of the sidechain	Reverse phase thin layer chromatography (undecane/ acetic acid:acetonitrile)	Sepn. of cholesterol (24-H)/ campesterol (24-methyl)/ and sitosterol (24-ethyl)	313
	Sephadex LH-20 gel column chromatography	Sepn. of 5β-cholestanol and 24-ethyl-5β-cholestanol	314
	"	Sepn. of cholesterol/campesterol/ β-sitosterol	315,316
	Digitonin fractional precipitation	Enrichment of short sidechain and low molecular weight sterols	310
Group separation according to stereochemistry of A/B ring junction	Silica gel thin layer chromatography	Sepn. of 5α/5β/Δ^5 sterols	314
Group separation according to unsaturation of the steroid nucleus or sidechain	Ag_2NO_3 impregnated silica gel or alumina thin layer chromatography	Sepn. of 5α/Δ^5 sterols as free compounds	313,317 318,319 320,321
	"	Sepn. of 5α/Δ^5 sterols as acetates	322,323
	Ag_2NO_3 impregnated silica gel column chromatography	"	309
	Sephadex LH-20 gel liquid chromatography	Sepn. of 5α/Δ^5/Δ^7 and Δ^8 sterols (5m x 1.6mm i.d.)	324

Table 3.2. (cont'd.)

		Fractionation of monoenes, dienes and trienes	325,326
Group separation of saturated from unsaturated species by chemical reaction of the double bonds.	Oxidation of double bonds and separation by column and thin layer chromatography		327,328 329,330
	Bromination of double bonds and separation by thin layer chromatography		331,332
	Epoxidation of double bonds and separation by thin layer chromatography	Sepn. of $5\alpha/\Delta^5$ sterols	333
Group separation of $5\alpha/\Delta^5$ compounds by oxidation of 3β-hydroxy group and separation of resulting ketones	Chemical oxidation of 3β-OH and separation by thin layer chromatography		334
	Enzymatic oxidation of 3β-OH and separation by TLC or g.c.		117,335 336
Analytical and preparative scale separations of sterols by molecular weight, size, or polarity	Lipophilic Sephadex gel liquid chromatography	Sepn. of $5\alpha/\Delta^5$ sterols and steroidal epimers at C-3, C-7, C-11, C-17 and C-20	337,338 339
	Reverse phase high pressure liquid chromatography	Sepn. of C_{27}, C_{28} and C_{29} sterol acetates with some selectivity for position of unsaturation	310,340

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where r_n is the retention time of the unsubstituted steroidal alkane and k_a, k_b etc are retention factors characteristic of the substituents and the number and position of olefinic bonds. Separation factors have been shown to be constant if all substituents and double bonds are electronically and stereochemically separated.[24,325,357] Separation factors for transformations of free sterols and sterol acetates of the common strcutural series on a variety of stationary phases have been recorded by Ikekawa[348] and Patterson,[349] respectively.

If equation 3.1. is based on the logarithm of retention time as with Kováts retention index,[253] then/the logarithms of separation factors become proportional to additive retention index increments. Using steroidal alkanes in place of n-alkanes in the semi-logarithmic relationship (e.g. 19 for androstane and 27 for cholestane) a steroid number system is created which has been shown to be less susceptible to temperature variations.[358,359] A number of other expressions have been proposed for steroid retention behaviour[359,360] but the Kováts retention index and simple relative retention have seen the most general application. The use of retention index data in the characterisation of sterols will be discussed in Section 3.3.3.

Sterol analysis by mass spectrometry is facilitated since the various structural types may be classified as members of highly branched homologous or quasi-homologous series. For example, in evaluating the mass spectra of sterols alkyl-substituted at position 24 in the side chain there will be common ions for the nucleus, whereas those ions retaining the side chain will show successive increases of fourteen mass units between members of the series. Similar considerations apply for alkylation within the steroid nucleus. Double bonds in the nucleus and sidechain, in addition to affecting the mass of molecular and fragment ions, may produce fragmentation characteristic of their position.

Mass spectra of many sterols as the free compounds[361,362] and various derivatives have been recorded and structure correlations discussed; acetates[275,295,363] trifluoroacetates,[361] methyl ethers[364,365] and trimethylsilyl ethers.[366-371] In seeking distinctions between closely similar isomers a comparison between two or more derivatives of differing mass spectrometric behaviour may be useful. Trimethylsilyl ethers of similar compounds are useful in the identification of common structural features but are often characterized by much non-distinctive fragmentation. In these circumstances mass spectra of the free compounds or of ester

derivatives of low mass spectrometric directing ability may serve to distinguish more subtle structural differences. For example, the mass spectra of the E and Z isomers of 24-alkylidene sterol trimethylsilyl ethers and the free compounds are dominated by structurally unspecific fragmentation. However, the acetate derivatives show consistent differences in the relative intensities of certain ions confirming the stereochemistry at C(28).[370] The diagnostic use of mass spectrometric fragmentation of sterol trimethylsilyl ethers for structure elucidation (with particular emphasis on double bond location) will be discussed in detail in Section 3.3.4.

The unique capabilities of combined gas chromatography-mass spectrometry in the analysis of complex mixtures have already been outlined (Section 1.4.). The wide occurrence of sterols in biological materials and the difficulty of obtaining pure compounds from these sources well suit g.c.-m.s. The advantages to be obtained by the use of glass open tubular columns of higher resolving power than the packed columns so far in general use are the theme of the present work.

3.2. Materials and Methods

3.2.1. Gas chromatography. Packed column gas chromatography was carried out on instruments equipped with flame ionization detectors (FID): Perkin-Elmer Model 881 and Carlo Erba Model GV. Columns were pyrex glass, nine feet and twelve feet, 3.5mm i.d., silanized and packed with 1% OV-1 coated on 100-120 mesh Gas Chrom Q (Phase Separations Limited, Queensferry, Clwyd, Great Britain). Carrier gas was nitrogen at 40ml/min.

Open tubular gas chromatography was carried out on a Silanox-type glass open tubular column (0.5mm i.d. x 50m) prepared according to the methods described in Section 2.1. and installed in a Pye Unicam Model 104 gas chromatograph equipped with FID according to the method described in Section 2.2.7.

3.2.2. Gas chromatography-mass spectrometry. Packed column g.c.-m.s. analyses were carried out on an LKB 9000 instrument equipped with a 6ft. (3.5mm i.d.) glass column packed with 1% OV-1 coated 100-120 mesh Gas Chrom Q (Phase Separations Limited, Queensferry, Clwyd, Great Britain). Helium carrier gas flow rate was 30ml/min. Open tubular g.c.-m.s. was carried out on a Silanox-type glass open

tubular column (0.5mm i.d. x 50m) prepared according to the method described in Section 2.1. and installed in the LKB 9000 according to the method described in Section 2.3. In both cases the electron energy was 70eV, emission current 60μA, ion source and separator temperature 270°C. Helium gas flow to the separator was 30ml/min.

3.2.3. Sterols. Sterol standards were obtained from the following sources: 5β-cholestan-3β-ol (Ikapharm, Ramat-Gan, Israel), 5-cholesten-3α-ol, 5α-cholestan-3α-ol (Steraloids Inc., Pawling, New York, U.S.A.), 5β-cholestan-3α-ol (K and K Rare and Fine Chemicals, I.C.N., Life Sciences Group, Plainview, New York, U.S.A.), 5-cholesten-3β-ol, 5α-cholestan-3β-ol (B.D.H. Chemicals Limited, Poole, England), 5α-ergost-7-en-3β-ol, 5α-ergost-8(14)-en-3β-ol, 5α-ergosta-7,22-dien-3β-ol, 5,7,22-(9β,10α)-ergostatrien-3β-ol (Dr. P. Bladon, Department of Chemistry, University of Strathclyde, Glasgow, Scotland) 5,22-ergostadien-3β-ol (Dr. M.J. Thompson, Insect Physiology Laboratory, U.S. Department of Agriculture, Beltsville, Maryland, U.S.A.), 5,7,22-ergostatrien-3β-ol (Dr. E.C. Horning, Institute of Lipid Research, Baylor College of Medicine, Houston, Texas, U.S.A.), 5α-ergosta-7,14,22-trien-3β-ol, 5α-ergosta-8(9),14,22-trien-3β-ol (Professor Sir Derek Barton, Imperial College of Science and Technology, University of London, England), 5,7,9(11),22-ergostatetraen-3β-ol (Dr. W.W. Reid, Institut Experimental du Tabac, Domaine De La Tour, Bergerac, France), 5,8(9),22-ergostatrien-3β-ol (A.R. Bader Collection, Aldrich Chemical Co. Ltd., Gillingham,Dorset, England).

Natural mixtures were obtained from the sources noted at the appropriate point in the text.

3.2.4. Preparation of derivatives. Trimethylsilyl ether derivatives of standards and mixtures of natural sterols were prepared in dry pyridine at 60°C for 30min using either hexamethyldisilazane and trimethylchlorosilane (HMDS/TMCS/4:1 v/v) or N-O-bis-(trimethylsilyl)-acetamide (BSA). Reagents were obtained from Pierce and Warriner (U.K.) Limited, Chester, Cheshire, CH1 4EF, England. Typically, approximately 500μg of sterol was taken up in 100μl of pyridine and 50μl reagent was added. After reaction, excess reagent and solvent were removed under a stream of dry nitrogen and the derivative was taken up in an appropriate volume of Nanograde hexane (concentration approximately 1mg/ml), particulate residues being removed by centrifugation.

Sterol samples obtained as acetate derivatives were subjected to

a mild microscale hydrogenolysis to afford the free sterol. Approximately 500µg of the derivative was treated with lithium aluminium hydride ($LiAlH_4$) in dry ether solution. After 30 minutes at room temperature excess reagent was quenched by cautious addition of ethyl acetate followed by a saturated aqueous solution of Rochelle Salt (sodium potassium tartrate). The resulting free sterols were extracted into ethyl acetate and derivatized in the normal way.

3.2.5. Calculation of retention indices. Kováts retention indices have been used to record the retention behaviour of the sterol trimethylsilyl ethers under isothermal chromatographic conditions. Indices obtained on packed columns have been produced by graphic semi-logarithmic interpolation between widely spaced co-injected n-alkane standards, usually octacosane ($n\text{-}C_{28}H_{58}$) and tetratriacontane ($n\text{-}C_{34}H_{70}$). Open tubular columns retention indices were obtained (either graphically or algebraically using Equation 1.6.) by reference to the appropriate bracketing, even-numbered n-alkanes.

It was found desirable to employ a relay technique for obtaining retention indices from the TIC chromatograms on the LKB 9000 using open tubular columns. With the somewhat longer analysis times associated with open tubular columns, instability of chromatographic conditions (primarily column temperature) makes it desirable to select internal standards which are eluted as close as possible to the components in question. The even numbered n-alkanes were used to obtain the retention index of the chosen internal standard, usually cholesterol TMS ether. Thereafter under the same conditions the retention time of cholesterol TMS included as standard or present itself in the natural mixture together with the slope of the n-alkane plotted line establish the standard with reasonable reliability even under moderately unstable conditions.

Retention index data obtained from literature values of relative retention times (RRT) may be readily obtained if the relative retention times of appropriate n-alkane are also recorded. The RRT of the chosen standard is simply a specified time or elution volume unit. RRT data without n-alkanes quoted may also yield retention indices provided comparison may be made with retention indices of at least two compounds in the body of retention index data already available. Under these circumstances graphic or algebraic calculations may be undertaken in the normal way. These techniques have been employed to compile the tabulated retention indices in Section 3.3.3. and Appendix A. Relay type calculations may somewhat obscure retention

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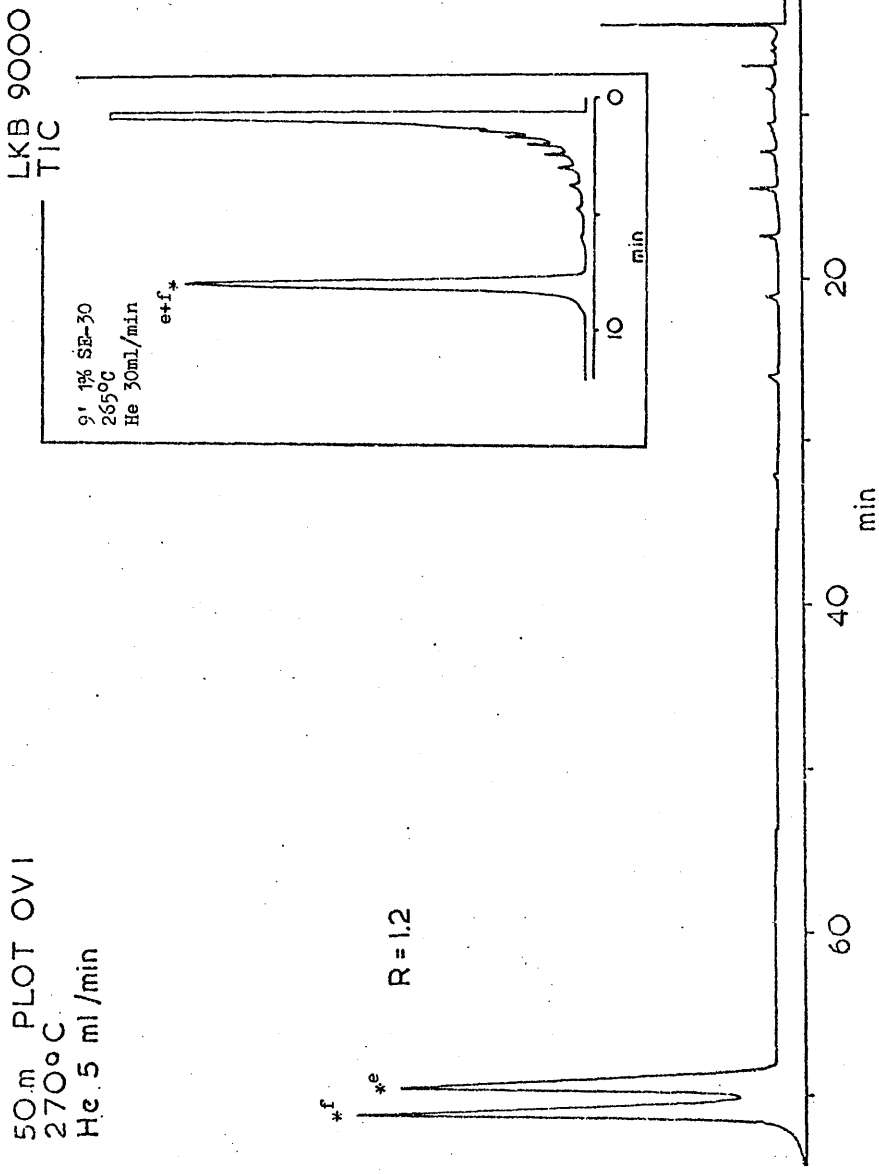


Figure 3.5. Total ion current (TIC) chromatogram of the separation on a 9ft. 1% SE-30 packed column (insert) and on 50m Silanox-type (PLOT) OV-1 column of 0.5ug, of a mixture (1:1) of cholesterol (e) and cholestanol (f) as trimethylsilyl ethers on the LKB 9000. Mass spectra were obtained at the points indicated (*) and these are represented in Figure 3.6.

index differences induced by differences in operating temperature. Where substantial differences in operating temperature (greater than $5^{o}C$) exist between two bodies of data, comparisons should be undertaken with some caution, the retention index varying about 1 index unit per ^{o}C.

3.3. Results and Discussion

3.3.1. Evaulation of open tubular g.c.-m.s. of sterol trimethylsilyl ethers. Separations of six sterols related to cholesterol were compared on packed and open tubular columns. The chromatographic data obtained are shown in Table 3.3. A comparison of the separation of a 1:1 mixture of cholesterol and cholestanol as trimethylsilyl ethers on both packed and open tubular columns installed in the LKB 9000 is shown in Figure 3.5. This separation may be considered a critical model in sterol analysis and the mass spectra obtained during these analyses are shown in Figure 3.6. The substantially unresolved packed column mass spectrum is shown in Scan 1 and the essentially pure components in Scans 2 and 3. Experience has shown that mass spectra obtained on packed or open tubular columns in g.c.-m.s. analysis are essentially identical. Some minor variations are observed, attributable to small variations in source temperature, source pressure or sample pressure. Figures 3.7. and 3.8. illustrate further model separations.

3.3.2. Open tubular g.c. and g.c.-m.s. of C_{28} sterols. Separations of eight C_{28} sterols related to ergosterol were compared on packed and open tubular columns. The chromatographic data obtained are shown in Table 3.4. These sterols differ in the number and position of double bonds in the nucleus and side-chain. This sort of diversity of structure, together with homologous compounds, characterizes the analysis of sterol mixtures of biological origin. Several model separations are compared on packed and open tubular columns in Figures 3.9. to 3.12. In these g.c.-m.s. analyses of standard mixtures there were few partial resolutions such as those pertaining to the isomeric cholestenes (Section 2.4.) which might allow a quantitative estimation of mass spectral purity obtaining for a given chromatographic resolution. However, these analyses generally conform to the behaviour demonstrated in Section 2.4.4., i.e. for a resolution of at least unity (R = 1) pure mass spectra are obtained

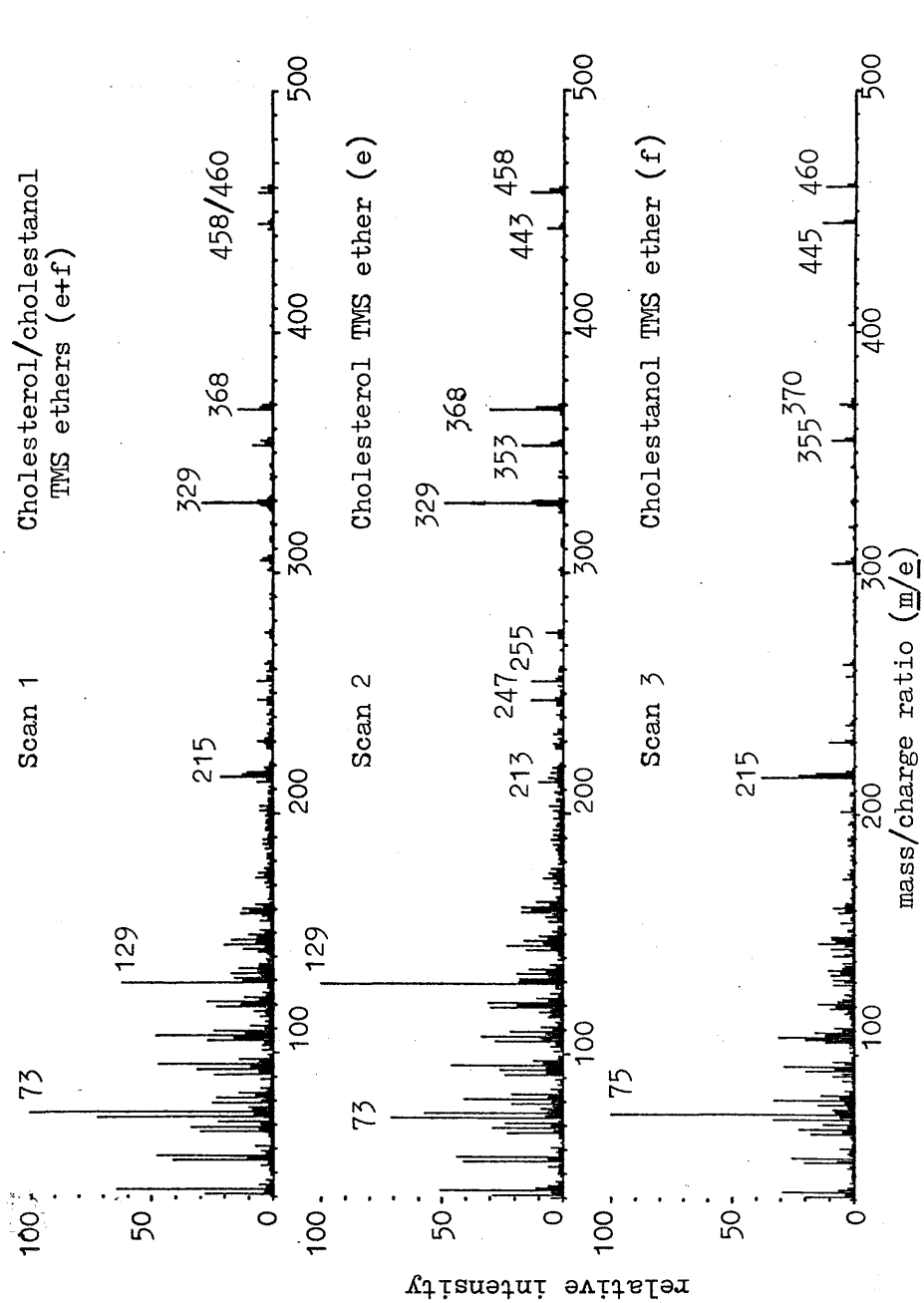


Figure 3.6. Mass spectra obtained in the g.c.-m.s. analyses on packed (Scan 1) and open tubular columns (Scans 2 and 3) of 0.5µg of a 1:1 mixture of cholesterol and cholestanol as trimethylsilyl ether derivatives. The columns were installed in the LKB 9000 in the normal way with source temperature 270°C and electron energy 70eV. TIC chromatograms and chromatographic conditions relevant to these analyses are represented in Figure 3.5.

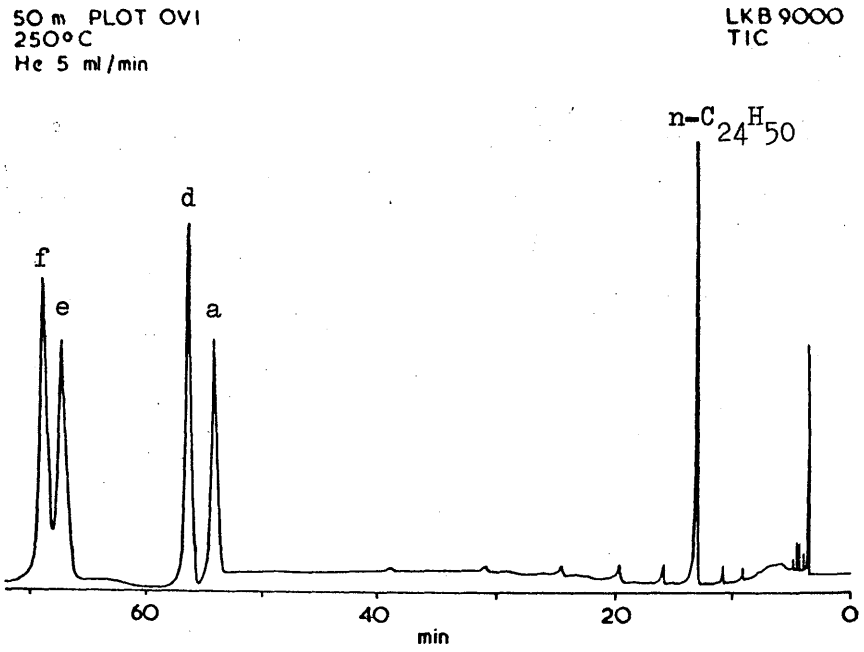


Figure 3.7. Total ion current (TIC) chromatogram of the separation of a mixture of coprostanol (a), epicoprostanol (d), cholesterol (e) and cholestanol (f) as trimethylsilyl ethers obtained on a 50m Silanox-type (PLOT) OV-1 column installed in the LKB 9000.

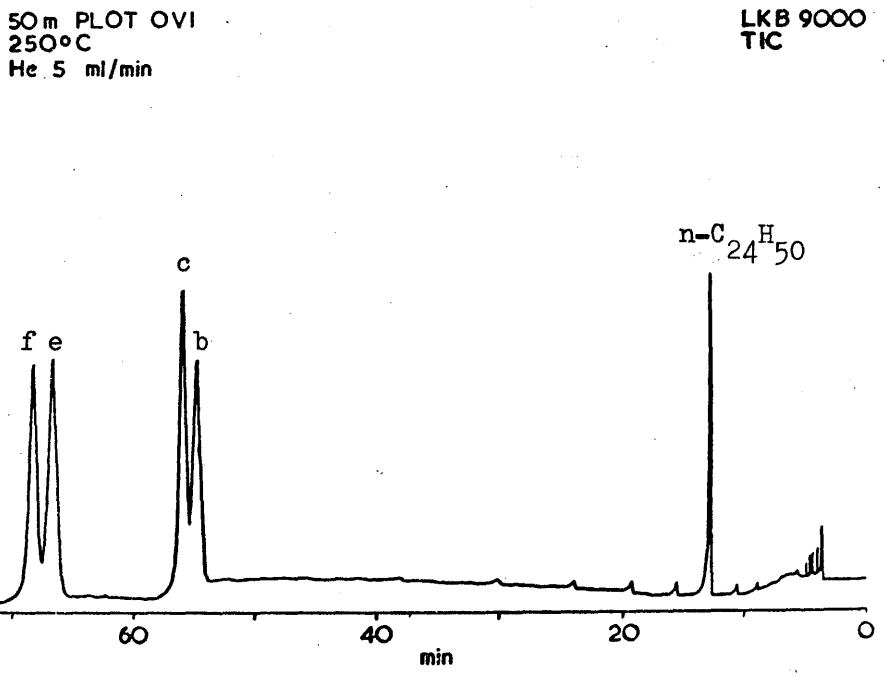


Figure 3.8. Total ion current (TIC) chromatogram of the separation of a mixture of epicholesterol (b), epicholestanol (c), cholesterol (e) and cholestanol (f) as trimethylsilyl ethers obtained on a 50m Silanox-type (PLOT) OV-1 column installed in the LKB 9000.

Table 3.3. Kováts retention indices for cholesterol, cholestanol and four analogues as trimethylsilyl ether derivatives on packed and and open tubular columns.

Figure reference *	Compound	Packed column** retention index $I^{OV-1}_{220^o}$	Open tubular column*** retention index $I^{PLOT\ OV-1}_{250^o}$
(a)	5β-Cholestan-3β-ol (coprostanol)	2887	3042
(b)	5-Cholesten-3α-ol (epicholesterol)	2898	3048
(c)	5α-Cholesten-3α-ol (epicholestanol)	3005	3055
(d)	5β-Cholestan-3α-ol (epicoprostanol)	3011	3064
(e)	5-Cholesten-3β-ol (cholesterol)	3042	3137
(f)	5α-Cholestan-3β-ol (cholestanol)	3045	3146

---

* Relevant chromatograms appear in Figures 3.5., 3.7. and 3.8.

** 9ft. 1% OV-1 column installed in Perkin-Elmer Model 881 gas chromatograph equipped with flame ionization detector, 220°C; carrier gas, nitrogen at 40ml/min. Indices calculated by semi-logarithmic interpolation of co-injected n-alkanes, $n\text{-}C_{28}H_{58}$ and $n\text{-}C_{34}H_{70}$.

*** 50m Silanox-type (PLOT) OV-1 column installed in the LKB 9000 at 250°C with He as carrier gas at 5ml/min. Indices calculated by relay based on cholestanol trimethylsilyl ether, I = 3146.

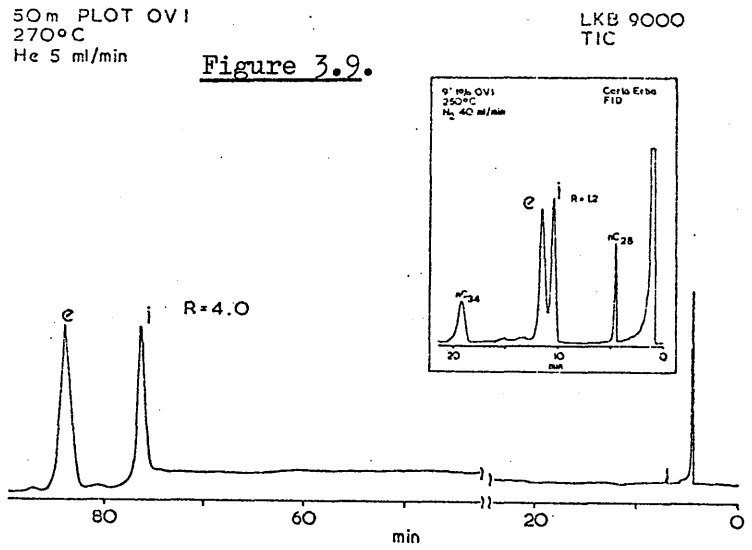


Figure 3.9.

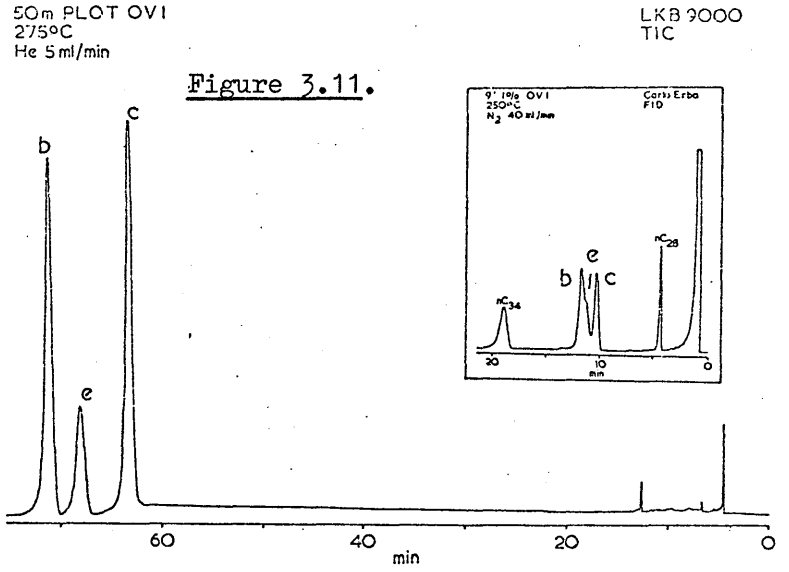


Figure 3.11.

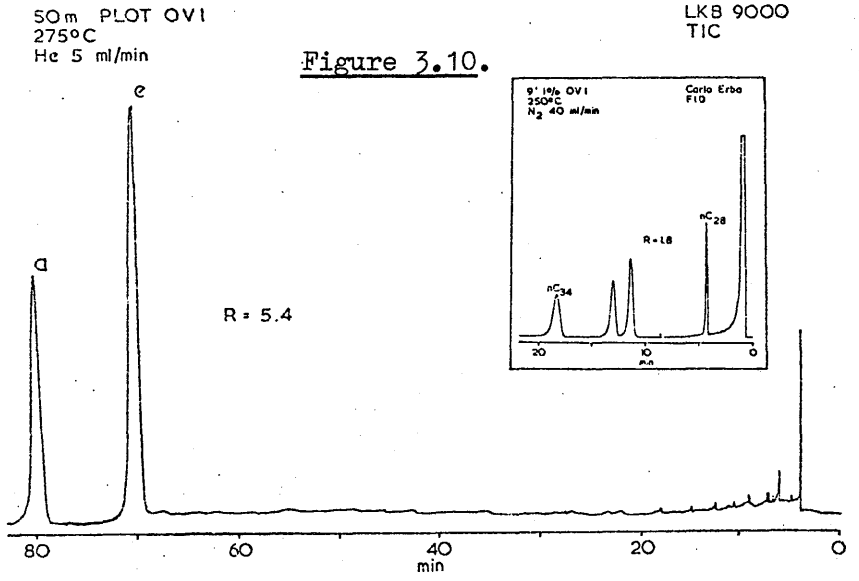


Figure 3.10.

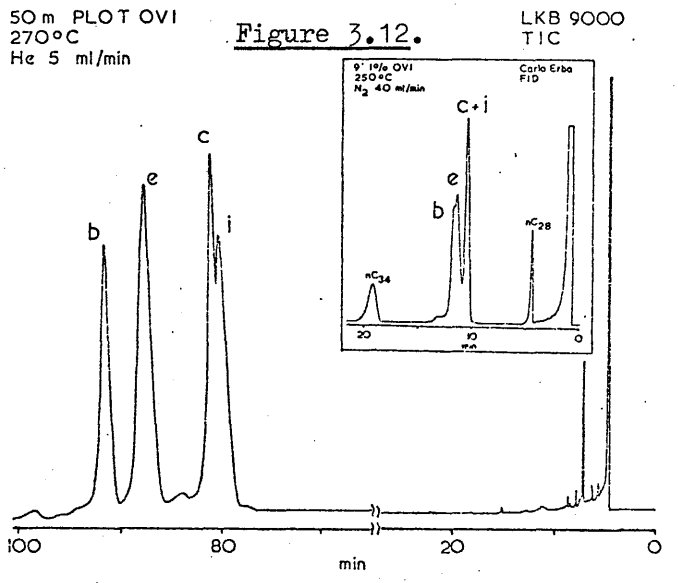


Figure 3.12.

Figures 3.9.-3.12. Separations of some synthetic sterol mixtures as TMS ether derivatives on glass open tubular and packed columns (inserts). Sterols referenced (a-i) and g.c. conditions as in Table 3.4.

Table 3.4. Kováts retention indices of unsaturated C_{28} sterol standards as trimethylsilyl ether derivatives on packed and open tubular columns.

Figure reference*	Compound	Packed column** retention index $I^{OV-1}_{250^oC}$	Open tubular column retention index** $I^{PLOT\ OV-1}_{265^oC}$
(a)	5α-Ergost-7-en-3β-ol	3263	3290
(b)	5α-Ergost-8(14)-en-3β-ol	3213	3242
(c)	5,22-Ergostadien-3β-ol (brassicasterol)	3169	3187
(d)	5α-Ergosta-7,22-dien-3β-ol	3208	3236
(e)	5,7,22-Ergostatrien-3β-ol (ergosterol)	3189	3225
(f)	5,7,22(9β,10α)-Ergostatrien-3β-ol (lumisterol)	3026	3041
(g)	5α-Ergosta-7,14,22-trien-3β-ol (ergosterol B3)	3148	3199
(h)	5α-Ergosts-8(9),14,22-trien-3β-ol (ergosterol B1)	3155	3203
(i)	5,7,9(11),22-Ergostatetraen-3β-ol	3156	3183

* Relevant chromatograms appear in Figures 3.9.-3.12.

** 12ft. 1% OV-1 column installed in a Carlo Erba gas chromatograph equipped with flame ionization detector, 250°C, carrier gas nitrogen at 40ml/min. Indices calculated by semi-logarithmic interpolation of co-injected n-alkanes, n-$C_{28}H_{58}$ and n-$C_{34}H_{70}$.

*** 50m Silanox-type (PLOT) OV-1 open tubular column installed in a Pye-Unicam Model 104 gas chromatograph equipped with flame ionisation detector , 265°C, with helium as carrier gas at 6ml/min. Indices obtained by semi-logarithmic interpolation between appropriate even numbered n-alkanes, co-injected.

3.3.3. Correlation of g.c. retention with sterol structure. As was mentioned earlier (Section 3.1.5.) the introduction of a functional group into a specific position in a molecule influences the retention time by a factor which is constant for the substituent and its particular site of attachment, but which is independent of the molecular weight of the total structure and of the influence of other non-interacting functional groups. This "group retention factor"[25,345,374] or "separation factor"[357] is of substantial analytical utility. Extension and refinement of these ideas have led to valuable correlations with structure for free sterols[348] and sterol acetates.[349] Retention changes produced by the preparation of derivatives[375] or by selective chemical modifications[376,377] have also been informative.

The Kováts system of retention index is useful for sterol analysis.[244] The data thus expressed are in a standard numerical form and are relatively invariable over a moderate temperature range (typically 1 index unit 1°C). Assessment of structure may be accomplished by comparison of absolute values of retention indices or by their increments. The argument for index increments is as for separation factors. Substitution in similarly constituted compounds increases the retention by the same amount for a given stationary phase.[253,378] Subject to the same qualifications, retention index increments may be employed, additively of course, in the same way as separation factors. That is, for isolated substituents the incremental effect of substitution is cumulative.

Table 3.5. incorporates the retention index data for unsaturated C_{28} sterol TMS ethers with retention indices from the literature or derived from relative retention data.[350,351,367,368] The table is constructed in a grid which facilitates comparison of various nuclear and side-chain alterations. While the data are from several methyl silicone stationary phases (SE-30, OV-1, or OV-101) and includes both packed and open tubular column data at several different temperatures there is a self consistency which offers useful correlations. Tables 3.6. and 3.7. summarize the retention index increments (dI) for several nuclear and side-chain transformations or substitutions. The retention index increments are identical to the "homorphy factor" (H) of Schomberg[378] or the "functional retention index" (FRI) of Swoboda.[379] These offer a means of characterizing the regularities of sterol retention behaviour.

A somewhat larger body of retention data on the behaviour of

Table 3.5. Correlation chart relating structure to retention index for some sterol trimethylsilyl ether derivatives on methyl-silicone stationary phase,*

Sidechain(*) / Nucleus	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1(a)	-	-	2930	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2(a)	3100	3070	3075	3135	-	3080	3130	-	-	-	-	3115	3200	3210	-	3215	-
(b)	3105	-	3100	3155	-	-	3150	-	-	-	-	-	-	-	3285	-	-
(c)	3140	-	3130	3180	-	-	-	-	-	-	-	-	3255	-	-	-	-
(d)	3145/3155	-	3133/3145	3185/3195[x]	-	-	-	-	-	-	-	-	-	-	-	-	-
3(b)	-	-	3060	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4(a)	-	-	-	-	3130	-	-	-	-	-	-	-	-	-	-	3200	-
(b)	-	-	3125	-	-	-	-	3010	-	-	-	-	-	-	-	-	3265
5(a)	-	-	3175	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(b)	-	-	3195	3235	-	3185	-	-	-	-	-	-	-	-	-	-	-
(c)	-	-	3225	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(d)	3250[x]	-	-	3290	-	3242	-	-	-	-	-	-	-	-	-	-	-
6(a)	-	-	-	-	-	-	3150	-	-	-	-	-	-	-	-	-	-
(b)	-	-	3140	3190	-	-	3170	-	-	-	-	-	-	-	-	-	-
(c)	-	-	3170	-	-	-	3210	-	-	-	-	-	-	-	-	-	-
(d)	3196	-	3187	3236	-	-	3225	3041	3199	3203	3183	-	-	-	-	-	-
7(b)	-	-	3380	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8(a)	3265	-	3260	-	-	-											
(b)	-	-	3280	-	-	-											
(c)	-	-	3305	-	-	-											
9(a)	-	-	3210	-	-	-											
(b)	-	-	3220	-	-	-											
(c)	-	-	3260	-	-	-											
10(a)	-	-	3270	-	-	-											
11(a)	-	-	3285	-	-	-											

* Source of data and GLC conditions as noted :

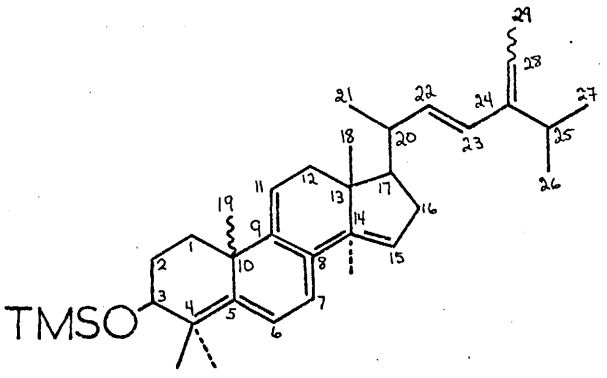
(a) C.J.W. Brooks, E.C. Horning and J.S. Young, Lipids, 3(5) (1968) 391-402, GLC conditions: 9ft 1% OV-1, 225°C, N_2 50ml/min.

(b) C.J.W. Brooks, W. Henderson and G. Steel, Biochim.Biophys. Acta, 296 (1973) 431-445, GLC conditions: 1.5m 1% SE-30, 230°C. N_2 40ml/min. Included are calculated indices obtained from RRT data in J.A. Ballantine, J.C. Roberts and R.J. Morris, J. Chromatogr., 103 (1975) 289-304, GLC conditions: 250°C.

(c) B.A. Knights, in Modern Methods of Steroid Analysis (E. Heftmann ed.) Academic Press, New York, 1973 pp. 103-138. GLC conditions: 1% OV-101, 255°C, N_2 40ml/min.

(d) Table 3.4 GLC conditions detailed therein.

Indices marked (x) have been calculated by semilogarithmic interpolation within the appropriate series. T = 250°C (underlined data T = 265°C).



COMPOSITE FORMULA

Nucleus

A C19-5α
B C19-Δ^4
C C19-Δ^5
D C19-Δ^7-5α
E C19-$\Delta^{8(9)}$-5α
F C19-$\Delta^{8(14)}$-5α
G C19-$\Delta^{5,7}$
H C19-9β,10α-$\Delta^{5,7}$
I C19-$\Delta^{7,14}$
J C19-$\Delta^{8(9),14}$
K C19-$\Delta^{5,7,9(11)}$
L C20-4β-methyl-Δ^5
M C20-4α-methyl-5α-Δ^7
N C21-4,4-dimethyl-Δ^5
O C22-4,4,14α-trimethyl-5α-Δ^7
P C22-4,4,14α-trimethyl-5α-$\Delta^{8(9)}$
Q C22-4,4,14α-trimethyl-5α-$\Delta^{9(11)}$

Sidechain

1 C7-24-nor-Δ^{22}
2 C8
3 C8-Δ^{22}
4 C8-$\Delta^{24(25)}$
5 C9-24-methyl
6 C9-24-methyl-Δ^{22}
7 C9-24-methylene-$\Delta^{24(28)}$
8 C10-24-ethyl
9 C10-24-ethyl-Δ^{22}
10 C10-24-ethylidene-$\Delta^{24(28)}$-E
11 C10-24-ethylidene-$\Delta^{24(28)}$-Z

Table 3.6. Retention index increments (dI) for double bond and stereochemical transformations in sterol trimethylsilyl ether derivatives.

Double Bond	Sterols compared	Carbon atoms nucleus(sidechain)	dI*
Nuclear double bond increments			
Δ^4	Δ^4/5α-stanol	$C_{19}(C_8)$	-30^a
Δ^5	Δ^5/5α-stanol	$C_{19}(C_8)$	-25^a,-5^b,-10^c,-10^d
		$C_{19}(C_{10})$	-5^a
	$\Delta^{5,22}/\Delta^{22}$-5α	$C_{19}(C_9)$	-9^d
Δ^7	Δ^7/5α-stanol	$C_{19}(C_8)$	$+35^a$,$+50^b$,$+40^c$,$+32^d$
		$C_{19}(C_9)$	$+40^d$
	$\Delta^{7,22}/\Delta^{22}$-5α	$C_{19}(C_9)$	$+50^d$
$\Delta^{8(14)}$	$\Delta^{8(14)}$/5α-stanol	$C_{19}(C_8)$	-20^a
Δ^{14}	$\Delta^{7,14,22}/\Delta^{7,22}$-5α	$C_{19}(C_9)$	-37^d
Δ^{16}	$\Delta^{5,16}/\Delta^5$	C_{19}	-40^{**}
	Δ^{16}/5α-stanol	C_{19}	-39^{**}
$\Delta^{5,7}$	$\Delta^{5,7}$/5α-stanol	$C_{19}(C_8)$	$+30^a$,$+25^b$,$+29^d$
Nuclear stereochemical increments			
5α → 5β	cholestanol/coprostanol	$C_{19}(C_8)$	-96^{***}
3α → 3β	epicholestanol/ cholestanol	$C_{19}(C_8)$	-91^{***}
9α,10β → 9β,10α	$\Delta^{5,7,22}$	$C_{19}(C_9)$	-184^d
Sidechain double bond increments			
Δ^{22}	$\Delta^{5,22}/\Delta^5$	$C_{19}(C_9)$	-55^c, -54^d
		$C_{19}(C_{10})$	-50^a,-60^b,-55^c
	$\Delta^{7,22}/\Delta^7$	$C_{19}(C_9)$	-45^b

Table 3.6. (continued)

$\Delta^{24(25)}$	$\Delta^{5,24(25)}/\Delta^{5}$	$C_{19}(C_8)$	-15^a
	$\Delta^{8(9),24(25)}/\Delta^{8(9)}$	$C_{22}(C_8)$	-15^a
$\Delta^{24(28)}$	$\Delta^{5,24(28)}/\Delta^{5}$	$C_{19}(C_9)$	-15^b
$\Delta^{24(28)}$-E	$\Delta^{5,24(28)}/\Delta^{5}$	$C_{19}(C_{10})$	-10^b
$\Delta^{24(28)}$-Z	$\Delta^{5,24(28)}/\Delta^{5}$	$C_{19}(C_{10})$	$+5^b$

Sidechain stereochemical increments

$\Delta^{24(28)}$E→Z	$\Delta^{5,24(28)}$	$C_{19}(C_{10})$	$+15^b$

---

* Increments obtained from g.c. data summarized in Table 3.5. superscript numbers referring to sources cited therein, except:
** Data cited in Table 2.6.
*** Data cited in Table 3.3.

Table 3.7. Retention index increments (dI) for alkyl substituents in sterol trimethylsilyl ethers.

Substituent	Sterols compared (carbon atoms)	Double bonds	dI*
24-Methyl	C_{27}/C_{28}	Δ^5	$+100^a$,$+95^c$
		Δ^7	$+95^d$
24-Ethyl	C_{27}/C_{29}	Δ^5	$+185^a$,$+175^d$
4α-Methyl	C_{28}/C_{27}	Δ^7	$+65^a$,$+75^d$
	C_{29}/C_{28}	Δ^5	$+95^a$
4β-Methyl	C_{28}/C_{27}	Δ^5	$+40^a$
4,4,14α-Trimethyl	C_{27}/C_{30}	Δ^7	$+130^a$
		$\Delta^{8(9)}$	$+70^b$

---

* Increments obtained from g.c. data summarized in Table 3.5. superscript numbers referring to soucres cited therein.

sterol acetate derivatives on a number of stationary phases is contained in papers by Patterson and co-workers.[349,373,381,382] The retention behaviour is recorded as RRT, but bracketing n-alkane relative retention times allow retention indices to be calculated. These are shown in Table A.1., Appendix A. Several of these papers contain RRT data obtained on the same g.l.c. systems under the same conditions. The retention indices derived from these provide a considerable volume of data of a highly self consistent nature. Together with some RRT/values of sterol acetates from other sources from which retention indices were obtained by interpolation are extensive correlations within a grid arrangement (Table A.2., Appendix A), which afford an extensive range of increments (dI) associated with double bond, stereochemical and alkylation transformations (Tables A.3.-A.5., Appendix A). These retention index increments on acetate and trimethylsilyl ether derivatives together show a number of correlations which may be useful for the evaulation of unknowns. The direct comparison between the two derivatives is, of course, confined to transformations remote from the site of derivatization. Some of these are discussed below.

With reference to the 5α-stanol TMS ether the corresponding Δ^5 compound is eluted 5-10 index units earlier on methyl-silicone stationary phases. On open tubular columns this is sufficient for baseline or near baseline resolution and in open tubular g.c.-m.s. mass spectra of the pure components are obtained (Section 3.3.1.). A somewhat better resolution (10 index units) is obtained for the acetate derivative. From the other comparisons available the introduction of other double bonds also decreases the retention time: $\Delta^{8(14)}$,-20 index units; $\Delta^{9(11)}$,-42 index units; Δ^{14},-37 index units; Δ^{16},-40 index units. Important exceptions to this trend are the Δ^7 and $\Delta^{5,7}$ systems which are retained to the extent of 25-50 index units. The conjugated $\Delta^{5,7}$ system must be considered as a single entity influencing the retention in a unique way, rather than representing the sum of the constituent Δ^5 and Δ^7 bonds. Each system of double bonds (di- or tri-unsaturated, conjugated or unconjugated) alters the stereochemistry of the nucleus in a particular way. While additive retention index increments may be useful, the evaluation of chromatographic behaviour must to some extent be considered on the individual case. Comparisons within the more extensive literature correlations on sterol acetates illustrate this.

Double bond alterations in the side-chain are relatively independent

of the double bond character (and of the derivative of the 3β-hydroxy group) in the nucleus. The introduction of a double bond at C(22)-C(23) reduces the retention time, the amount depending on the cis and trans configuration and the alkyl substitution at C(24). For sterol acetates the order of elution of double bond isomers is $\Delta^{22} < \Delta^{24(25)} < \Delta^{24(28)}$. The effect of a double bond at the 24(28) position, compared to the saturated system, is small: -15 index units. The two possible isomers of the 24-ethylidene system have opposing effects when compared to the saturated C_{10} side-chain: E-24(28),-10 index units; Z-24(28),+5 index units. The difference between the 24-ethylidene E and Z isomers is 15 index units, sufficient for a complete resolution and distinction on glass open tubular columns.

The effect of alkyl substitution varies according to the position and unsaturation of the parent sterol. The addition of a methyl group at C(24) produces a nearly ideal homologous increment (+90-100 index units). The second methyl group is also effective (24-ethyl, +185 index units). The presence of 4α- or 4β-methyl groups does not increase the retention as much as those above, partly as a result of interactions with the 3-acetoxy or 3-trimethylsilyloxy groups. This effect forms the basis of a g.c. method for distinguishing between 4,4-dimethyl, 4-methyl and 4-demethyl sterols.[349,375] The effect of stereochemical interactions may also be seen in the fact that the 4α-methyl increment is slightly higher for 5α- and Δ^5-sterols than for the corresponding Δ^7 and $\Delta^{8(9)}$ compounds.

The behaviour of the 14α-methyl groups is unexpected. For the 4,4,14-trimethylsubstitution the dI is +130 index units for a Δ^7 sterol trimethylsilyl ether and only +70 for a $\Delta^{8(9)}$. This is more dramatically demonstrated in the sterol acetate correlations (Table A.2., Appendix A). The addition of a 14α-methyl group in a Δ^7 sterol increases the retention by only +15 index units and for the $\Delta^{8(9)}$ the order of elution is actually reversed (-2.9 index units).

Additional increments may be extracted from these data and those obtained from unsaturated steroidal hydrocarbons (Section 2.4.2. Table 2.8.). Probable retention indices may be estimated for compounds not available or for comparisons with unknown data.

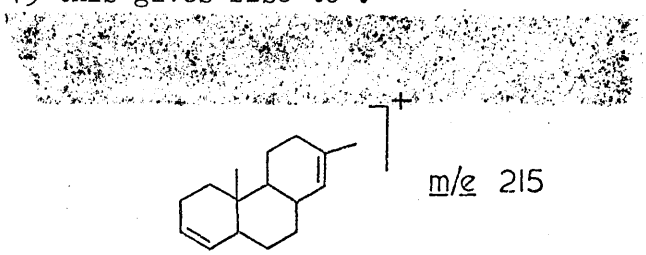
3.3.4. Correlation of structure and m.s. fragmentation in sterol trimethylsilyl ethers. The mass spectra of the standard sterol TMS ethers listed in Table 3.4. were obtained by packed column g.c.-m.s. on

the LKB 9000. These data are summarized in Table 3.8. and the full line diagrams appear in Appendix B.

The mass spectra of some of these and many other sterols as TMS ether derivatives have been investigated and useful diagnostic features identified (References 361 and 366-371 and references cited therein). The literature taken with the current data are discussed below. Some prominent ions of the mass spectra of sterol TMS ethers and their origin are summarized in Table 3.9. In this table each process discussed is given an indexing letter and this letter may be quoted in text or tabular data as shorthand reference for a specific ion (e.g. for m/e 353 arising from the loss of a methyl group and the elements of trimethylsilanol, with the word "fragmentation" shortened to "frag.", the text may be shortened to m/e 353 (frag. c) or in tabulated data entered as 353^{c}).

The mass spectra of sterol trimethylsilyl ethers are generally characterized by prominent molecular ions. Nonspecific ions characteristic of the trimethylsilyloxy moiety are also prominent: m/e 73, $(CH_3)_3Si^+$; m/e 75, $(CH_3)_2Si{=}\overset{+}{O}H$ and loss of the elements of trimethylsilanol, $[M-90]^{\ddagger}$ (frag. b). Loss of a methyl group, $[M-15]^+$, (frag. a) is usually also abundant and may derive either from the trimethylsilyloxy group or from the steroid nucleus. The former origin is demonstrated by the prominence of $[M-18]^+$ ions in the spectra of d_9-TMS ether derivatives and the latter by the prominence of an ion at $[M-(90+15)]^+$, (frag. c) in the spectra of TMS ether derivatives of many sterols. These latter three ions (frag. a, b and c) may be useful as indirect indicators of the mass of $M^{\ddagger}$.

(a) Mass spectra of stanol TMS ethers. Fully saturated 5α-3β-hydroxy-steroid TMS ethers show many ions of comparable abundance, most prominent among these being the loss of trimethylsilanol, side-chain and C(15) through C(17) of ring D and one proton from the nucleus (frag. q'). Where the nuclear methyl substitution is only at C-10 and C-13 this gives rise to :



m/e 215

Table 3.9. Some structurally significant ions in the mass spectra of sterol trimethylsilyl ethers.

Ion	Structure or genesis	Fragmentation reference	Comments
$M^{\cdot+}$	molecular ion		molecular weight
m/e 43	$C_3H_7^+$		non-specific
m/e 73	$(CH_3)_3Si^+$		"
m/e 75	$(CH_3)_2Si=\overset{+}{O}H$		"
$[M-15]^+$	$M^{\cdot+}-CH_3$	a	Indirect molecular weight, more prominent in systems with conjugation.
$[M-90]^{\cdot+}$	$M^{\cdot+}-(CH_3)_3SiOH$	b *	
$[M-105]^+$	$M^{\cdot+}-[(CH_3)_3SiOH+CH_3]$	c *	
m/e 129	$[(CH_3)SiO=CH-CH=CH_2]^+$	d	Δ^5-3-OTMS, Scheme 2.4.
$[M-129]^+$	$M^{\cdot+}-[(CH_3)_3SiO=CH-CH=CH_2]$	e	
m/e 131	$(CH_3)_3SiO=CH-CH_2-CH_3^+$	f	$\Delta^{5,7}$-3-OTMS, Scheme 3.1.
$[M-131]^+$	$M^{\cdot+}-[(CH_3)_3SiO=CH-CH_2-CH_3]$	g	
[a][M-43]+	$M^{\cdot+}-C_3H_7$	h	Cleavage of terminal isopropyl group in sidechain.
$[M-133]^+$	$M^{\cdot+}-[C_3H_7+(CH_3)_3SiOH)]$	h'	
[b][M-(‖C24 +2)]+	$M^{\cdot+}$-[sidechain from C(24)+2xH]	i	Cleavage adjacent to Δ^{22} in sidechain.
$[M-(‖C24 +2+90)]^+$	$M^{\cdot+}$-[sidechain from C(24)+2xH+$(CH_3)_3SiOH$]	i'	
[c][M-(‖C23 +1)]‡	$M^{\cdot+}$-[sidechain from C(23)+1xH]	j	McLafferty rearr. of $\Delta^{24(28)}$, Scheme 3.2. also seen in spectra of Δ^{22}. -$\Delta^{5,24(28)}$ compounds.
$[M-(‖C23 +1+90)]^{\cdot+}$	$M^{\cdot+}$-[" " C(23)+1xH+$(CH_3)_3SiOH$]	j'	
$[M-(‖C23 +1+15)]^+$	$M^{\cdot+}$-[" " C(23)+1xH+CH_3]	k	
$[M-(‖C23 +1+15+90)]^+$	$M^{\cdot+}$-[" " C(23)+1xH+CH_3+$(CH_3)_3SiOH$]	k'	
$[M-(‖C23 +1+129)]^{\cdot+}$	$M^{\cdot+}$-[" " C(23)+1xH+$(CH_3)_3SiO=CH-CH=CH_2$]	l	
[d][M-(‖C22 +2)]+	$M^{\cdot+}$-[sidechain from C(22)+1xH]	m	Δ^{22}, Scheme 3.5.
$[M-(‖C22 +1+90)]^{\cdot+}$	$M^{\cdot+}$-[" " C(22)+1xH+$(CH_3)_3SiOH$]	m'	
$[M-(‖C22 +2)]^+$	$M^{\cdot+}$-[" " C(22)+2xH]	n	
$[M-(‖C22 +2+90)]^+$	$M^{\cdot+}$-[" " C(22)+2xH+$(CH_3)_3SiOH$]	n'	
[e][M-SC]+	$M^{\cdot+}$-sidechain	o **	Sidechain fragmentation: transformations of $[M-90]^+$ distinctive of sidechain unsaturation:
$[M-(SC+90)]^+$	$M^{\cdot+}$-[sidechain + $(CH_3)_3SiOH$]	o' **	
[f][M-(SC+27)]‡	$M^{\cdot+}$-[" + (C16-C17)]	p	****
$[M-(SC+27+90)]^{\cdot+}$	$M^{\cdot+}$-[" + (C16-C17)+$(CH_3)_3SiOH$]	p'	
[g][M-(SC+42)]+	$M^{\cdot+}$-[" + (C15-C17)]	q	
$[M-(SC+42+90)]^+$	$M^{\cdot+}$-[" + (C15-C17)+$(CH_3)_3SiOH$+1xH]	q' ***	
$[M-(SC+1)]^{\cdot+}$	$M^{\cdot+}$-[" +1xH]	r	Saturated, Δ^{22} -> o'> t' Δ^{24}, $\Delta^{24(28)}$, Δ^{25} -> t'> o'
$[M-(SC+1+90)]^{\cdot+}$	$M^{\cdot+}$-[" +1xH+$(CH_3)_3SiOH$]	r' *****	
$[M-(SC+1+15)]^+$	$M^{\cdot+}$-[" +1xH+CH_3]	s	
$[M-(SC+1+15+90)]^+$	$M^{\cdot+}$-[" +1xH+CH_3+$(CH_3)_3SiOH$]	s'	
$[M-(SC+2)]^+$	$M^{\cdot+}$-[" +2xH]	t	Note: frag. t favoured in cases of sidechain unsaturation.
$[M-(SC+2+90)]^+$	$M^{\cdot+}$-[" +2xH+$(CH_3)_3SiOH$]	t'	
[h][M-(SC+2+42+90)]+	$M^{\cdot+}$-[" +2xH+(C_{15}-C_{17})+$(CH_3)_3SiOH$]	u	
m/e 69		v	-Δ^{22} and Δ^{24}, most prominent in latter.

a) Cleavage C(24)-C(25) of side-chain.
b) Cleavage C(23)-C(24) of side-chain.
c) Cleavage C(22)-C(23) of side-chain.
d) Cleavage C(20)-C(22) of side-chain.
e) Cleavage of entire side-chain, C(17)-C(20).
f) Fragmentation of ring D involving loss of unit consisting of C(16)-C(17), 27 mass units or 26 mass units(with appropriate H transfer).
g) and h) Fragmentation of ring D involving loss of unit consisting of C(15)-C(17), 42 mass units.

* Proportions indicative of stereochemistry at C(5).
** Prominent side-chain fragments for saturated side-chain.
*** Base peak in stanol TMS ether spectra.
**** Ring D fragments promoted by C(8) unsaturation.
***** Base peak or prominent ion in $\Delta^{14,22}$ sterol TMS ethers.

Mono- or disubstitution at C(4) shifts the resulting fragment ion by 14 or 28 mass units, respectively. Where the substitution is at C(14), deductions from this ion may be less reliable since this substituent may itself be eliminated. In the fully saturated nucleus there is little direct fragmentation of ring A. Further indications about 4-alkylation may be obtained from the intensities of m/e 107 (for 4,4'-demethyl) m/e 121 (for 4-monomethyl) and m/e 135 (for 4,4'-dimethyl).[371] The exact origin of these ions has not been investigated and the fragmentation of ring A may be dramatically influenced by unsaturation in the nucleus.

Various stereochemistries at positions 3 and 5 in stanols may easily be distinguished by gas chromatography (Section 3.3.1.). The mass spectra are also informative. The principal feature distinguishing 3β- and 3α-epimers is the strong peak at m/e 108 characteristic of the former.[366,382] Further small differences may be accentuated by the recording of spectra at low electron energy. 5β-compounds exhibit a base peak at $[M-90]^{+\cdot}$ (frag. b) and a low intensity molecular ion.[367] A general rule differentiating these epimers on the basis of the relative intensities of the $[M-90]^{+\cdot}$ (frag. b) and $[M-105]^{+}$ (frag. c) ions has been found useful.[353] This states that the 5β-stanol TMS ether should exhibit intensities of $[M-90]^{+\cdot}$ greater than $[M-105]^{+}$ (frag. b > frag. c) and 5α-stanols the reverse (frag. b < frag. c). Or more reliably, for the 5β-stanol TMS ethers $[M-90]^{+\cdot}$ must exceed twice the intensity of $[M-105]^{+}$ (frag. b > 2·frag. c).

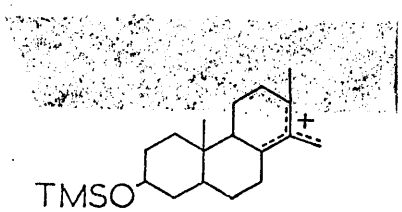
The further important naturally occurring epimeric pair arises from the R and S configuration of the 24-alkyl substitution in the sterol side-chain. As has been mentioned earlier, mass spectrometry and gas chromatography have so far failed to be useful in making this distinction.

(b) Mass spectra of nuclear unsaturated sterol TMS ethers

Δ5-stenol TMS ethers. In the mass spectra of Δ^5 unsaturated sterol TMS ethers the two strongest peaks are generally m/e 129 (frag. d) and $[M-129]^{+}$ (frag. e). The genesis of these ions have already been discussed (Section 2.3.8.). In 4-alkylcholesterol derivatives the $[M-129]^{+}$ is further enhanced.[367] However, deductions of structure based on these ions may be equivocal.[383] Other structural types, notably 2β-trimethylsilyloxy steroids, 17-trimethylsilyloxy steroids[367] and $\Delta^{9(11)}$-3β-trimethylsilyloxy steroids,[368] may afford mass spectra

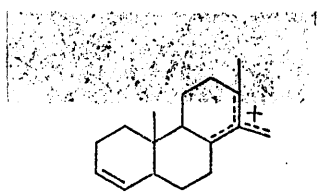
in which ions of m/e 129 are prominent. The $[M-129]^+$ ion is much more reliable in these cases being prominent only in the Δ^5-3β-trimethylsilyloxy compounds.

Δ^7-, $\Delta^{8(9)}$- and $\Delta^{8(14)}$-stenol TMS ethers. Mass spectra of these three sterol structures show many similar characteristics. They show prominent molecular ions, which may be the base peak and also strong ions at $[M-15]^+$ (frag. a) and $[M-105]^+$ (frag. c). In addition there are prominent fragment ions arising from the loss of the side-chain and a two carbon unit from ring D.[367] For a 4,14-demethyl sterol these are:



m/e 318

frag p



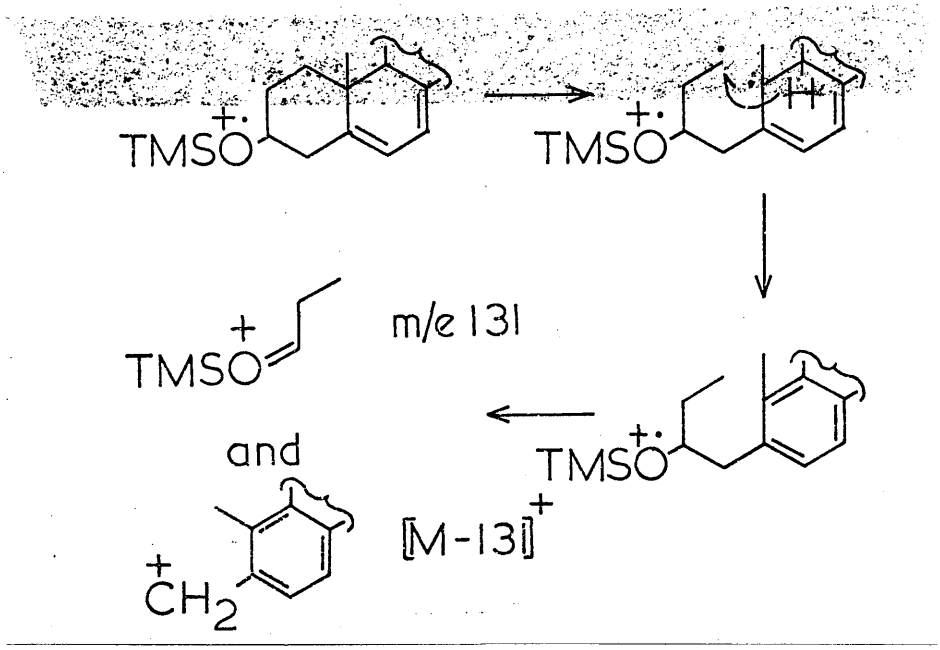
m/e 229

frag p'

Ions arising from a complete loss of ring D (frag. q and frag. q') may also be prominent. It has been suggested that the occurrence of common ions in the spectra of these three double bond isomers is the result of the production of a common intermediate under electron impact prior to fragmentation.[384] A further important mass spectral distinction occurs in the case of the 14α-alkyl-substituted compounds in which the elimination of this methyl group, $[M-15]^+$ (frag. a) is particularly intense in $\Delta^{8(9)}$ compounds.[371]

9,10-cyclopropano-sterol TMS ethers. A distinctive ion in the spectrum of cycloartenol TMS ether arises from the 9,10-cyclopropane ring-directed elimination of C_3H_7 from the $[M-90]^+$ ion. This transition is supported by metastable ion evidence.[371]

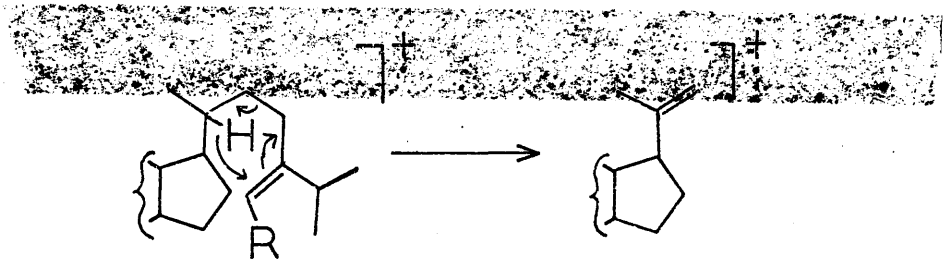
$\Delta^{5,7}$-stenol TMS ethers. Sterol TMS ethers of the conjugated $\Delta^{5,7}$ structure give particularly characteristic mass spectra. Together with a prominent molecular ion and $[M-105]^+$ (frag. c), which usually affords the base peak of the spectrum these also show abundant ions at m/e 131 (frag. f) and $[M-131]^+$ (frag. g). These ions which characterise the $\Delta^{5,7}$ system may be rationalized by Scheme 3.1.[367] Ions at m/e 129 and $[M-129]^+$ which might have been expected from the Δ^5-3-trimethylsilyloxy structure are of low abundance and another prominent ion may appear at m/e 144 arising from the trimethylsilyloxy group plus a three carbon fragment.



Scheme 3.1.

(c) Mass spectra of side-chain unsaturated sterol TMS ethers. Side-chain saturated and unsaturated sterol TMS ethers in addition to other more characteristic and diagnostic fragmentations may occasionally show moderate amounts of ions at $[M-43]^+$ (frag. h) and $[M-90-43]^+$ (frag. h') and a prominent m/e 43 among the low mass ions. This may be rationalized as a cleavage of the C(24)-C(25) bond.

$\Delta^{24(28)}$ sterol TMS ethers. Sterols containing unsaturation at $\Delta^{24(28)}$ show a typical fragmentation involving a McLafferty-type rearrangement (frag. j).[385,386] This ion may be rationalized by the following scheme.



R = H		$[M-84]^{+\cdot}$
R = CH_3		$[M-98]^{+\cdot}$
R = C_2H_5		$[M-112]^{+\cdot}$

Scheme 3.2.

The intensity of this characteristic fragment may be influenced by the nature of the steroid nucleus (e.g. low abundance in the case of $\Delta^{8(9),24(28)}$ structures[371]).

The cleavage of the side-chain, and of the side-chain plus the elements of trimethylsilanol, are both accomplished with a transfer of two hydrogen atoms from the nucleus to the departing radical (frag. t and frag. t', respectively). However, this hydrogen transfer does not function exclusively in the case of elimination from the $[M-90]^{+\cdot}$ ion and in this case a process without hydrogen migration (frag. n') predominates.

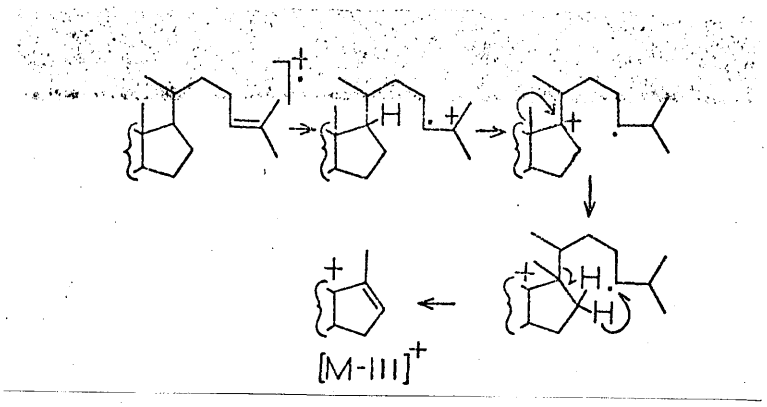
The assignment of configuration of the 24-alkylidene side-chain, 24(28)-E and 24(28)-Z is not possible in the mass spectra of the TMS ethers. However, when considered simultaneously with chromatographic data, minor but diagnostic differences occur in the mass spectra of the acetate derivatives. In the spectra of the isomeric pairs of 24-ethylidene-Δ^7-sterolacetates ions corresponding to $M^{+\cdot}$, $[M-15]^+$, $[M-98]^{+\cdot}$ $[M-(139+60)]^+$ and m/e 55 are consistently stronger in relation to the base peak, $[M-141]^+$ (frag. j) in the E-series than in the Z-series.[370] This is consistent with the hypothesis that in the E-series the McLafferty rearrangement is slightly hindered, the $[M-141]^+$, (frag. j) making a weaker contribution relative to other competitively formed ions.[386]

Δ^{22}- and Δ^{24}-sterol TMS ethers. The fragmentation associated with the loss of the unsaturated side-chain has been extensively studied by Wyllie and Djerassi.[387] Using deuterium labelling experiments it was found that two fragmentation mechanisms compete, either simple loss of the side-chain via allylic cleavage of the C(17)-C(20) bond or loss of the side-chain plus two additional hydrogens. The schemes proposed for the latter process which proceed by different mechanisms in Δ^{22} and Δ^{24} components are shown below.

For sterol TMS ethers a comparison of the intensities of ions arising from the loss of trimethylsilanol plus side-chain (frag. o') with ions from loss of trimethylsilanol, side-chain plus 2 hydrogens (frag. t') readily distinguished Δ^{22} from Δ^{24} and Δ^{25}. The former predominates in the Δ^{22} compounds, the latter in Δ^{24} and Δ^{25} structures. In contrast, in analogous fragmentations not associated with the loss of trimethylsilanol the loss of two hydrogen-atoms (frag, t) is favoured in Δ^{22}, Δ^{24} and Δ^{25} compounds.

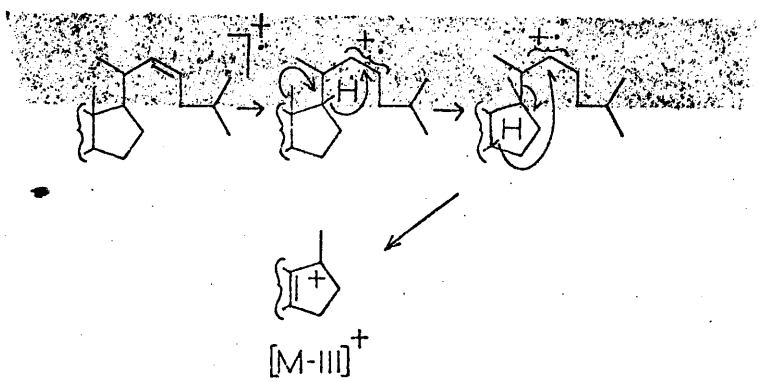
In these compounds a number of less diagnostic ions arise from

Δ^{24}-steroids



Scheme 3.3.

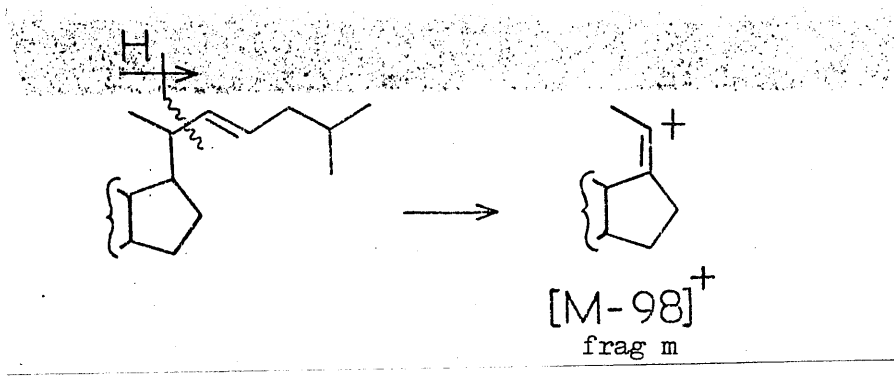
Δ^{22}-steroids



Scheme 3.4.

the fragmentation of ring D resulting in the elimination of the side-chain and a unit consisting of C(16)-C(17) or the side-chain and C(15)-C(17) with and without loss of trimethylsilanol. These are associated with a numerical decrement in the mass of the nuclear fragment (in addition to the loss of side-chain and trimethylsilanol) of 27 (or occasionally 26 with a single hydrogen transfer) and 42 mass units respectively. In the case of side-chain unsaturation this is generally accompanied by a transfer of two protons to the departing radical.[361] However, the mass and abundance of these ions depends not only on the nature of the side-chain but also on the substitution and unsaturation in the nucleus.

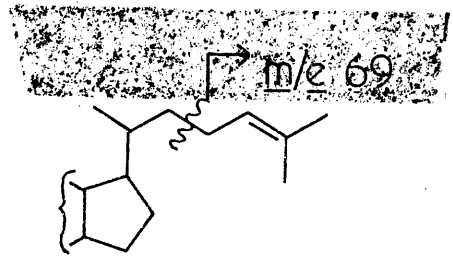
In the case of Δ^{22} structures an apparently unfavourable cleavage of the C(20)-C(22) bond with the transfer of a single proton to the departing radical results in an ion of modest intensity, (frag. m), e.g.:



Scheme 3.5.

This may also be associated with concurrent loss of 90 mass units (frag. m') and both processes may be accompanied by an additional proton migration (frag. n and frag. n', respectively). Cleavage adjacent to the C(22)-C(23) bond on the other side with a two proton transfer to the nuclear fragment (frag. i and frag. i') may also be apparent.

In the Δ^{24} structures the low mass ion at m/e 69 often supersedes all others in the mass spectrum. This may be rationalized as the allylic cleavage of the side-chain between C(22) and C(23).



frag v

However, this indication should be viewed with circumspection since this ion is also prominent among the low mass fragments of Δ^{22} sterol TMS ethers.

Multiple conjugated unsaturation in the side-chain, in common with the behaviour of nuclear conjugation, tends to stabilize the structure, enhancing the molecular ion and suppressing fragmentation which may be structurally informative. The loss of side-chain in $\Delta^{22,24(28)}$ sterol TMS ethers arises only from the ion resulting from the elimination of trimethylsilanol. These ions appear with and without the transfer of two protons, the latter predominating.[371]

3.3.5. Evaluation of standard spectra. Standard mass spectra of the compounds listed in Table 3.4. are summarized in Table 3.8. These may

Table 3.8. Salient features of the mass spectra of unsaturated C_{28} sterols as trimethylsilyl ether derivatives obtained at electron energy 70eV.

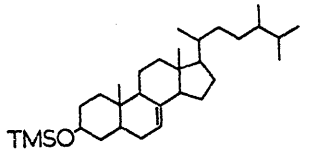
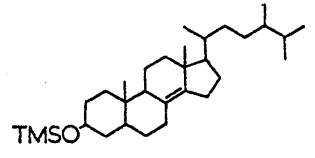
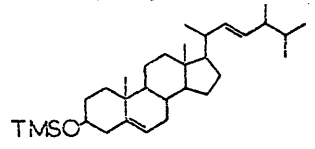
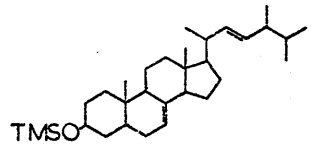
Figure reference*	Compound	Prominent ions (indexed according to Table 3.9.)						
(a)	5α-ergost-7-en-3β-ol $C_{19}'(C_9)$  TMSO $C_{31}H_{56}OSi$ (472)	472 (74)	$255^{o'}$ (100)	75 (130)*	55 (60)	107 (59)	81 (53)	95 (48)
		73 (46)	105 (45)	57 (42)	$213^{q'}$ (40)	$229^{p'}$ (33)	382^{b} (10)	345^{o} (9)
		318^{p} (4)						
(b)	5α-ergost-8(14)-en-3β-ol $C_{19}'(C_9)$  TMSO $C_{31}H_{56}OSi$ (472)	472 (88)	107 (100)	75 (124)*	73 (76)	55 (67)	147 (59)	57 (54)
		105 (50)	81 (50)	$229^{p'}$ (50)	$213^{q'}$ (43)	$255^{o'}$ (35)	345^{o} (11)	382^{b} (11)
(c)	5,22-ergostadien-3β-ol $C_{19}'(C_9')$  TMSO brassicasterol $C_{31}H_{54}OSi$ (470)	470 (14)	129^{d} (100)	125 (96)	69^{v} (204)*	55 (104)*	73 (83)	$255^{o'}$ (39) 83 (39)
		105 (28)	380^{b} (25)	343^{t} (17)	341^{e} (14)	215 (12)	397^{a} (8)	372^{j} (4)

Table 3.8. (cont'd.)

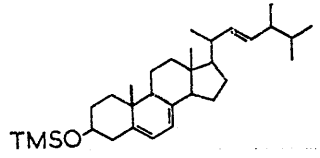
(d) 5α-ergosta-7,22-dien-3β-ol
$C_{19}'(C_9')$



$C_{31}H_{54}OSi$
(470)

470 (40)	255[o] (100)	69[v]* (200)*	75* (200)*	73* (140)*	81* (113)*	107 (88)
95 (66)	343[t] (63)	105 (63)	67 (57)	229[p'] (56)	125 (33)	372[j] (13)
					380[b] (10)	337[h'] (7)

(e) 5,7,22-ergostatrien-3β-ol
$C_{19}''(C_9')$

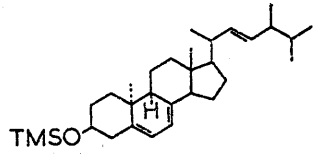


ergosterol

$C_{31}H_{52}OSi$
(468)

468 (32)	363[c] (100)	69[v]* (160)*	73* (150)*	131[f] (63)	119 (55)	337[g] (72)
143 (47)	253[o'] (38)	211 (37)	157 (36)	378[b] (25)	125 (22)	

(f) 5,7,22-(9 ,10)-ergostatrien-3β-ol
$C_{19}''(C_9')$



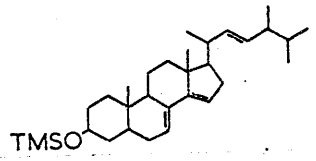
lumisterol

$C_{31}H_{52}OSi$
(468)

468 (26)	253[o'] (100)	69[v]* (115)*	55* (100)*	363[c] (58)	159 (58)	81 (55)
143 (52)	343[o] (35)	378[b] (20)	337[h'] (19)	125 (11)	453[a] (2)	

Table 3.8. (cont'd.)

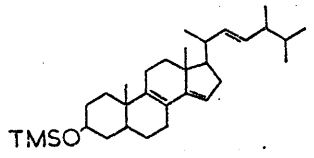
(g) 5α-ergosta-7,14,22-trien-3β-ol
$C_{19}"(C_{9}')$



ergosterol B3 — $C_{31}H_{52}OSi$ (468)

468 (30)	342[r] (100)	69[v] (30)	81 (41)	453[a] (33)	370[j] (20)	107 (17)	
129 (13)	131 (10)	145 (10)	253[o] (10)	378[b] (3)			

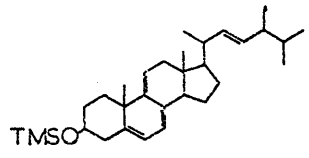
(h) 5α-ergosta-8(9),14,22-trien-3β-ol
$C_{19}"(C_{9}')$



ergosterol B1 — $C_{31}H_{52}OSi$ (468)

468 (61)	342[r] (100)	69[c] (56)	363[c] (43)	453[b] (32)	237[s] (29)	182 (28)	143 (22)
91 (20)	129 (21)	159 (17)	105 (17)	131 (16)	145 (15)	253[o'] (14)	171 (12)

(i) 5,7,9(11),22-ergostatetraen-3β-ol
$C_{19}"(C_{9}')$



$C_{31}H_{50}OSi$ (466)

466 (7)	251[o'] (100)	69[v] (108)*	73 (70)	55 (49)	376[b] (37)	209[q'] (8)
249[c'] (17)	361[c] (8)	451[a] (3)				

* Spectrum normalised on the most intense peak above m/e 100

be rationalized as follows:

(a) The mass spectrum of 5α-ergost-7-en-3β-ol TMS ether was found to be in good agreement with previously recorded data.[368,371] The base peak of the spectrum, m/e 255, is due to the elimination of the side-chain plus trimethylsilanol (frag.o'). A notable feature, characteristic of the saturated side-chain is the loss of the side-chain without net hydrogen transfer, m/e 345 (frag.o).[367,484] Further characteristic ions arise from the degradation of ring D: m/e 318 (frag.p), m/e 229 (frag.p') and m/e 213 (frag.q').

(b) The mass spectra of $\Delta^{8(14)}$ sterol TMS ethers are very similar to those of the corresponding Δ^{7}-components[367,368,371] but the isomers are easily distinguishable by gas chromatography. Minor differences are observed and are of diagnostic value: m/e 255 (frag.o') and m/e 213 (frag.q') are less prominent in the $\Delta^{8(14)}$ sterol.

(c) The spectrum of brassicasterol TMS ether is in agreement with data recorded elsewhere.[367,368] The presence of the Δ^{22} bond induces useful diagnostic ions. The true base peak of the spectrum is m/e 69. Cleavage adjacent to the double bond with charge retention on the nuclear fragment gives rise to m/e 372, $[M-98]^{+}$ (frag.j). An ion at m/e 125 arises directly from the unsaturated side-chain. Prominent ions arising from side-chain cleavage plus 2 nuclear hydrogen atoms transferred, m/e 343 (frag.t) and loss of the side-chain terminal isobutyl group plus trimethylsilanol, m/e 337 (frag.h') are likewise evidence of side-chain unsaturation. The base peak of the spectrum is m/e 129 (frag.d) and is accompanied by the corresponding $[M-129]^{+}$, m/e 341 (frag.e).

(d) 5α-ergosta-7,22-dien-3β-ol TMS ether shows abundant ions at m/e 255 (frag.o') and m/e 229 (frag.p') whose prominence characterizes the Δ^{7} structure. The mass spectrum also contains ions characteristic of the Δ^{22} side-chain unsaturation: m/e 343 (frag.t). m/e 227 (frag. h'), m/e 372 (frag.j) and m/e 69 (frag.v).

(e) The fragmentation of ergosterol TMS ether has been discussed elsewhere.[367,371] The $\Delta^{5,7}$ system gives rise to characteristic ions at m/e 131 (frag.f) and m/e 337, $[M-131]^{+}$ (frag.g). An interesting ion at m/e 211 seems to comprise ring D with side-chain attached. The unsaturated side-chain and the elements of trimethylsilanol are eliminated in m/e 253 (frag.o').

(f) Lumisterol TMS ether - as expected for a stereoisomer - shares many of the characteristics of ergosterol TMS ether. However, the base peak has altered to m/e 253 (frag.o') and an ion characteristic

of the loss of the unsaturated side-chain, m/e 343 (frag. o) is prominent.

(g) The mass spectrum of 5α-ergosta-7,14,22-trien-3β-ol TMS ether shows a strong molecular ion and $[M-15]^+$. An ion arising from the loss of side-chain and a single proton forms the base peak, m/e 342 (frag. r). This is in contrast to the behaviour of ring D saturated Δ^{22} compounds which show no transfer. This might be explained by the promotion of hydrogen transfer from C(16) by the Δ^{14} double bond and is observed in other $\Delta^{14,22}$ compounds.[369] Other prominent ions in the spectrum are m/e 370 (frag. j) arising from the cleavage of the side-chain adjacent to the Δ^{22} bond and m/e 253 (frag. o) arising from the loss of side-chain and trimethylsilanol.

(h) The spectrum of 5α-ergosta-8(9),14-22-trien-3β-ol TMS ether shares some characteristics with the trienol previously discussed. There are a prominent molecular ion and $[M-15]^+$ ion. The base peak, m/e 342 (frag. r), is formally from side-chain cleavage accompanied by a single hydrogen transfer. The loss of the elements of trimethylsilanol plus the side-chain, m/e 253 (frag. o'), shows no hydrogen migration from the nucleus. The one-proton transfer is also operating in ions associated with methyl loss: m/e 327, $[M-(\text{side-chain}+15+H)]^+$ (frag. s) and m/e 237, $[M-(\text{side-chain}+15+90+H)]^+$ (frag. r'). These ions are observed in the 24-ethyl homologue.[369] In the case of the side-chain saturated system the Δ^{14} bond exercises no substantial influence on side-chain cleavage.[324,338] A further prominent fragment at m/e 182 may be rationalised as a fragmentation across ring B and is a feature of other $\Delta^{8(9)}$ sterol TMS ether mass spectra.[371]

(i) In the mass spectrum of 5,7,9(11),22-ergostatetraen-3β-ol TMS ether the most prominent high mass ion (base peak above m/e 100) is associated with the side-chain: m/e 251, M -(side-chain+90), (frag. o'), conforming to the expectation of Δ^{22} systems. The true base peak of the spectrum is m/e 69. An ion arising from the fragmentation of ring D is prominent: m/e 209 $[M-(\text{side-chain}+C(15)-C(17)+90)]^+$ (frag. q'). The molecular ion and simple fragmentations make up the remaining intense ions in the spectrum. The extended conjugation in the nucleus tends to suppress fragmentation.

3.3.6. Conclusions. Where ambiguities arise in the identification of sterols by gas chromatography or mass spectrometry the application of the combined technique is advantageous. The model "difficult" sterol g.c. separation chosen in the early stages of this work, the 5α-Δ^5

isomeric pair, exemplifies the advance in facility in g.c. and g.c.-m.s. Mixtures of comparable proportions of these compounds may easily be estimated by m.s. alone. In highly disproportionate mixtures, difficulties arise which the baseline resolution on glass tubular columns (afforded by the 5-10 index unit difference) in g.c.-m.s. analysis easily resolves.

A less well defined m.s. distinction such as the 24-ethylidene E - Z (approximately 15 index units difference) makes combined g.c.-m.s. with columns of high efficiency essential. While the opportunity for a direct test in this study did not arise the distinction should be categorical. Likewise the unambiguous identification of the three double bond possibilities at C(8) in the nucleus should be possible. Whereas the Δ^{7} isomer is well separated (by packed column g.c.) from the $\Delta^{8(9)}$ and $\Delta^{8(14)}$[367] the distinction between the latter two is less marked: 20 index units in the acetates (see Table A.2., Appendix A). This difference should be sufficient where 10 index units represents a baseline separation and permits the recording of pure mass spectral data.

The separation of the R and S epimers of 24-alkyl sterols (e.g. stigmasterol and poriferasterol) has been unsuccessful. The chromatographic resolution of these compound types is, of course, critical for an unambiguous assignment, as their mass spectrometric behaviour is apparently identical.[368] Small but measurable retention differences for 24-R-S epimeric pairs have been reported by other workers.[348,349] These so far remain unconfirmed. Proton magnetic resonance at high field (220MHz) is the method of choice in this distinction.[389,390]

A liquid-solid chromatographic technique has been shown to be effective in the resolution of 24-alkyl sterane epimers.[148] This has interesting implications for other chromatographic techniques; however, where sample polarity, complexity of mixtures or limited quantities of sample indicate the need for a gas phase analysis, the configuration at C(24) must at present remain unassigned.

The extent to which an improvement in chromatographic efficiency is efficaceous in a particular analysis is a question of the sample itself. In mixtures of sterols differing in degrees of unsaturation and alkylation the opportunities for fortuitous overlaps are numerous and unpredictable (see Figure 3.12.). Further advances in the utility of g.c.-m.s. for sterol analysis rest on the broadening of the power of the gas chromatography. An increase in efficiency is the

first, most obvious improvement. While the glass open tubular columns described here are a significant improvement on packed column performance they are modestly efficient by modern standards of open tubular column performance (other workers have reported efficiencies of 150,000 theoretical plates). Likewise the columns utilized here are of a single apolar phase, a phase reckoned by Patterson to be the most generally useful for sterol analysis.[349] Open tubular columns of other, more polar, phases would provide a range of separation modes which would be useful for complex mixtures.

In addition to dI values on a single phase ΔI values, retention index differences on a polar and an apolar phase at a specified temperature, have been recognized as structurally significant parameters.[378,391] This two-dimensional chromatographic correlation has been applied to several classes of compounds (see: 392 and references therein) and open tubular columns of different polarities have been incorporated into tandem chromatographic systems of high instrumental refinement and flexibility.[393]

With increased resolving power the precision with which retention data are determined is more important. At column efficiencies of 50- to 100-thousand theoretical plates a retention index difference of 1-2 units is measurable and potentially significant. To make these differences quantitatively reliable careful control is required for column polarities, column temperature and flow rate. The LKB 9000 system is particularly subject to variation in the last two parameters.

Columns of higher resolving power of polar and apolar phases operated under conditions allowing precision of the highest practicable degree would significantly augment the analytical advantage demonstrated here with glass open tubular columns. The use of these columns on sterol mixtures from various sources is the subject of the following section.

3.4. Applications in Sterol Analysis

3.4.1. Marine sterol mixtures. Marine invertebrates usually possess complex mixtures of C_{25}, C_{26}, C_{27}, C_{28} and C_{29} 4-demethyl sterols, often with 5α- and the corresponding Δ^5-analogues.[395] Over 80 sterols have so far been identified from marine sources[286,395] and evidence has been presented for the occurrence of over 30 sterols in a single marine species.[396] As the catalogue of novel sterols grows so also does the necessity for refined methods of separation. The

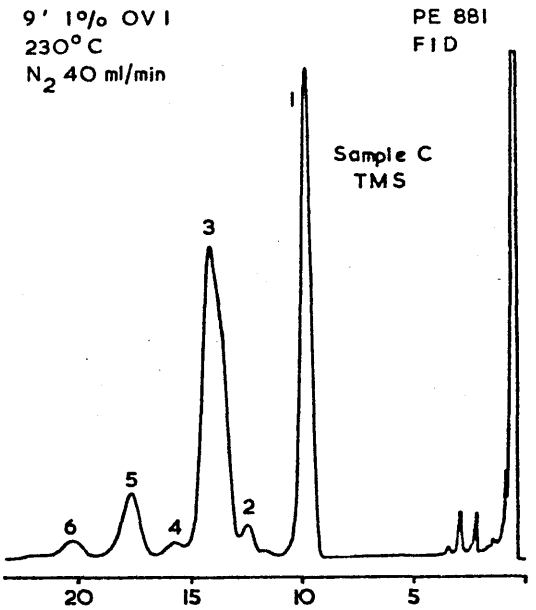


Figure 3.13. Packed column separation of the sterols (fraction M-1-1) of the scallop Placopecten magellanicus as trimethysilyl ether derivatives.

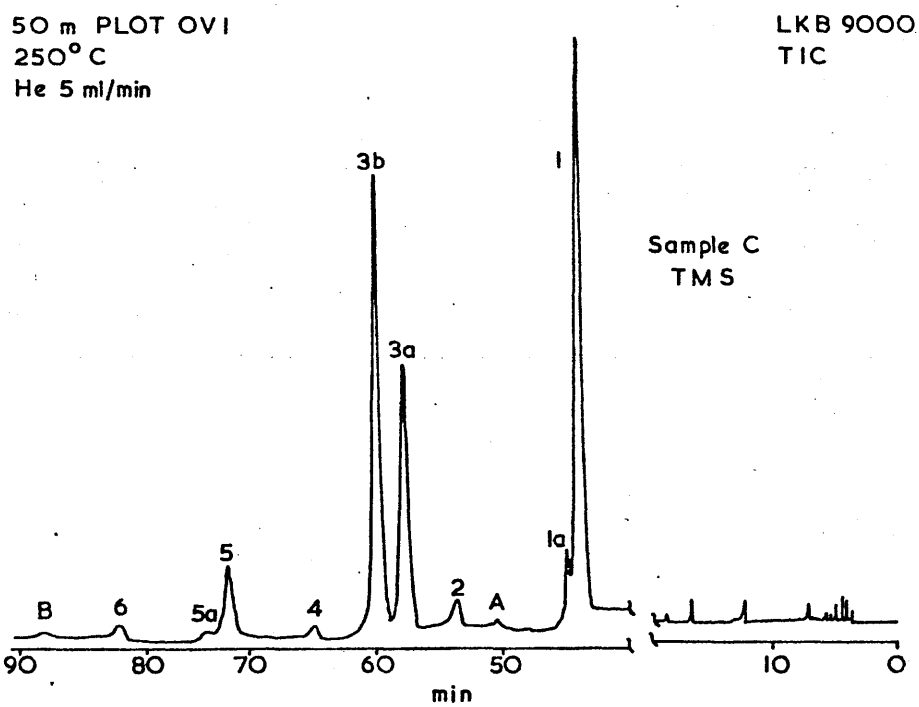


Figure 3.14. TIC chromatogram of the separation on a 50m Silanox-type (PLOT) OV-1 column installed in the LKB 9000 fraction M-1-1, sterols of the scallop Placopecten magellanicus as trimethylsilyl ether derivatives.

emphasis by workers has so far been on the isolation in preparative quantities of the mixtures following preliminary examination by packed column g.c. G.c.-m.s. has been one of several methods of characterisation applied after considerable preliminary work. Analysis by packed column g.c. and g.c.-m.s. after only a minimum of sample preparation has proved only partially satisfactory.[397] Analysis with columns of higher resolving power is thus of great importance, particularly where the quantities of sample are so small as to allow only a minimum of manipulation.

As the number of recognised variations of sterol nucleus and side-chain increases, the cases where these "non-classical" sterols constitute the major components of the extracts of marine animals remain relatively few. Methods of analysis of the components of mixtures over a wide range of concentrations with emphasis on the minor constituents is an area of growing interest.[310] The endogenous origin of these minor sterols as intermediates or active metabolites is of biosynthetic interest, particularly in comparative terms. An exogenous origin of these compounds implies that these are the major sterols of organisms lower in the marine food chain. Thus their analysis is of descriptive biochemical, chemotoxonomic and ecological importance.

(a) Sterols of the scallop Placopecten magellanicus. The mixture of sterols of this mollusc indigenous to the Gulf of St. Lawrence was the subject of an extensive fractionation procedure involving Sephadex LH-20 gel liquid chromatography, column chromatography on Anasil B and silver nitrate t.l.c. by Professor D. Idler and co-workers at the Marine Sciences Research Laboratory, Memorial University of Newfoundland, St. John's, Newfoundland, Canada.[339] Fractions were subject to long packed column g.c. (9m) and g.c.-m.s. (7m) on SE-30 to good effect.[352] A fraction obtained from Sephadex LH-20 chromatography (M-1-1) was examined by g.c.-m.s. on a 50m glass open-tubular column. Significant advantage was obtained over packed columns (compare Figures 3.13. and 3.14) and the g.c.-m.s. results, shown in summary in Table 3.10, confirmed those already obtained.

The higher resolution available had several informative consequences: (a) A minor component eluting 5 index units after peak 1 (24-nor-5,22-cholestadien-3β-ol) showed mass spectral features consistent with the corresponding 5α-analogue m/e 444, M^{+}; m/e 257 (frag.o'); m/e 374 (frag.m), m/e 345 (frag.t). These features are represented in the partial mass spectra shown in Figure 3.15.

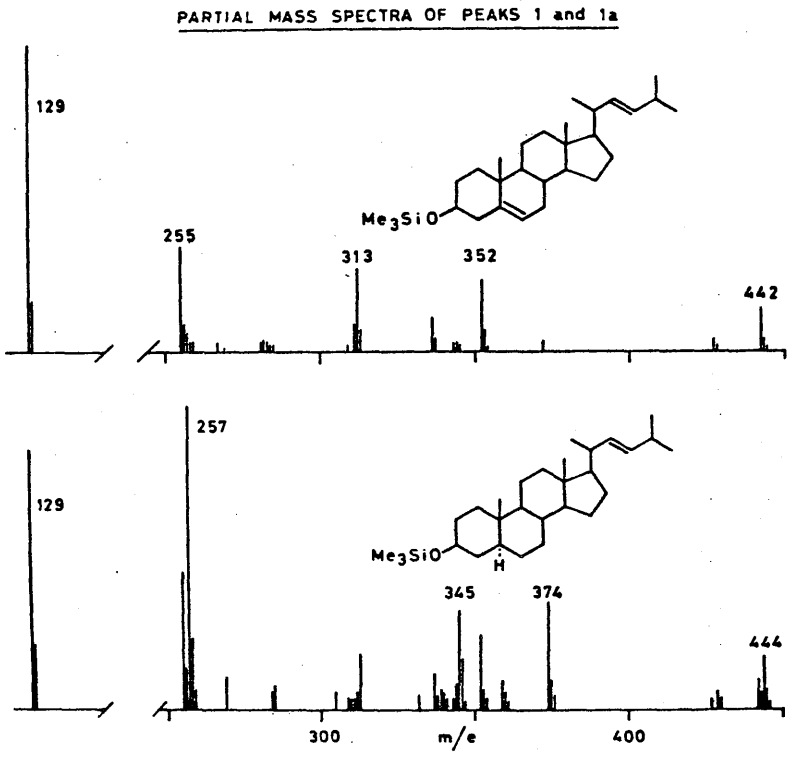


Figure 3.15. Partial mass spectra obtained of the sterols 1 and 1a in fraction M-1-1 of sterols of the scallop Placopecten magellanicus as trimethylsilyl ether derivatives. Structures inferred from gas chromatographic data and mass spectral features illustrated are shown. Mass spectra were obtained on the LKB 9000 with a source temperature at 270°C and electron energy 70eV. The TIC chromatogram of this analysis is shown in Figure 3.14.

(b) The cis-22- and trans-22-isomers of 5,22-cholestadien-3β-ol are resolved to near baseline (dI = 10 index units) and the pure mass spectra obtained were found to be identical, (c) A minor component insufficient to provide an interpretable mass spectrum was observed at $I^{250^{o}}_{OV-1}$ = 3200 (peak 5a) eluted ten index units after brassicasterol (peak 5) indicating a possible 5α-analogue.

Table 3.10. Retention indices and solvent mass spectrometric data for components of Fraction M-1-1 as trimethylsilyl ethers.

Figure reference*	$I^{275^{o}}_{OV-1}$**	M^{+} (%)	Base peak m/e	Other ions m/e
1	2995	442 (5)	97	129,352,313
1a	3000	444 (15)	257	347,97,374
2	3075	456 (5)	111	129,255,372
3a	3105	456 (10)	111	129,255,327
3b	3115	456 (11)	111	129,255,327
4	3150	458 (10)	129	329,368,255
5	3190	470 (17)	129	125,341,380
6	3230	470 (4)	129	341,380,386

* Figure 3.13. and 3.14.

** Long packed column, 7m 1% SE-30, He 30ml/min, 275°C, open-tubular resolutions calculated by relay (i.e. 1a and 3a,3b). Key to components: (1) 24-nor-5,22-cholestadien-3β-ol; (1a) 24-nor-5α-cholest-22-en-3β-ol; (2) isomer of 5,22-cholestadien-3β-ol (20-iso or modified side-chain?); (3a) 5,22-cis-cholestadien-3β-ol; (3b) 5,22-trans-cholestadien-3β-ol; (4) cholesterol; (5) 24-methyl-22-dehydrocholesterol; (6) 24-methylenecholesterol.

(b) Sterols of the molluscs of Class Amphineura : Placiphorella velata (Sample G) and Ischnochiton sp. (Sample H). Samples of the sterols of these two chitons were obtained from Dr. Idler with the request to confirm the major components: 5-cholesten-3β-ol, 5α-cholestan-3β-ol and 5α-cholest-7-en-3β-ol. The TIC chromatograms for the g.c.-m.s. analysis of the major components are shown in Figure 3.16. and the g.c. and m.s. data confirming these identities appear in Table 3.11. Some of the difficulties associated with instability in the g.c. conditions on the LKB 9000 are demonstrated in the retention indices shown there.

These results are consistent with the identification of 5α-cholest-

Table 3.11. Gas chromatographic and mass data obtained on the major constituents of Samples G and H and standard compounds as trimethylsilyl ether derivatives in the g.c.-m.s. analysis on the LKB 9000 with open tubular columns.

Figure* reference	$I_{250°C}$ PLOT OV-1	M^+*** (%)	Base peak	Prominent ions (%)	Comments
	3133**	458 (13)	129	353,329,301,107 (17)(44)(3)(37)	standard cholesterol
	3145**	460 (32)	215	355,305,107 (24)(24)(83)	standard cholestanol
	3185**	458 (66)	255	353,229,213,107,105 (17)(35)(48)(78)(58)	standard 5α-cholest-7-en-3β-ol
D. Idler Sample G					
a	3133***	458 (10)	129	353,329,301,107 (18)(54)(2)(48)	cholesterol
b	3145	460 (35)	215	355,305,107 (30)(30)(85	cholestanol
c	3188***	458 (66)	255	353,229,213,107,105 (18)(36)(71)(66)(40)	5α-cholest-7-en-3β-ol
D. Idler Sample H					
a	3133***	458 (11)	129	353,329,301,107 (15) (48)(2) (40)	cholesterol
b	3145	460 (30)	215	355,305,107 (28) (30)(80)	cholestanol
c	3194***	458 (62)	255	353,229,213,107,105 (15) (35)(60)(70)(50)	5α-cholest-7-en-3β-ol

* Relevant chromatograms appear in Figure 3.16.
** Indices calculated by semi-logarithmic interpolation with co-injected n-alkanes, n-$C_{30}H_{62}$ and n-$C_{32}H_{66}$.
*** Indices calculated by a relay technique based on cholestanol TMS ether I = 3145.
**** Mass spectrometer conditions: ion source temperature 270°C and electron energy 70eV.

7-en-3β-ol in other species of chiton[398,399] and have interesting parallels in biosynthetic investigations on other marine organisms. Teshima and co-workers[399] have demonstrated the synthesis of 5α-cholest-7-en-3β-ol from mevalonate without incorporation into cholestanol in the chiton, Liolophura japonica. On the other hand, Smith and Goad demonstrated the synthesis of 5α-cholest-7-en-3β-ol from cholesterol via cholestanol in two species of starfish.[400]

Attempts to obtain data on the minor constituents of these samples are shown in Figure 3.17. These were largely unsuccessful due to the large divergence in concentration between the major and the minor constituents ($>10^2$). Due to the low levels of minor constituents it was necessary to overload the column to the detriment of column performance. Additional difficulties were experienced in the "memory" of major constituents at high m.s. sensitivity, even for well resolved compounds. Table 3.12. summarises the g.c. and m.s. data obtained. The degree of reproducibility of operating parameters affected the reliability of retention indices.

The minor constituents of Placiphorella velata (Sample G) suggested by g.c. and m.s. are 5α-cholesta-7,22-dien-3β-ol, 24-methyl-5,22-cholestadien-3β-ol and 24-ethyl-5-cholesten-3β-ol. The second of these is corroborated by independent g.c. examination at Memorial University.[401] In the case of Ischnochiton sp. (Sample H) there were more numerous minor constituents at lower concentrations and the overload of major constituents adversely affected chromatographic performance. By comparison with the results of Sample G, the retention increments (Table 3.6.) and standard retention indices (Table 3.5. , in particular the data of Knights[351]) evidence for 5,22-cholestadien-3β-ol, 5α-cholesta-7,22-dien-3β-ol, 24-methyl-5,22-cholestadien-3β-ol and 24-ethyl-5-cholesten-3β-ol was obtained. The low intensity scans from which the evidence for minor constituents are extracted (Table 3.12.) are complicated by numbers of artifactual ions arising from background and cross-contamination of overloaded major constituents. For example, m/e 191 appears as an intense ion and might be attributable to carbohydrate TMS ether contamination arising from the original LH-20 chromatography. Identification of these compounds is tentative and offered without detailed discussion.

(c) Sterols of the sponge Axinella polypoides. Professor L. Minale and Dr. G. Sodano at the Laboratorio par la Chemica e Fisica di Molecole di Interesse Biologico del C.N.R., Naples, Italy, studied the total sterols of the sponge Axinella polypoides collected from the Bay

Table 3.12. Gas chromatographic and mass spectral data obtained on the minor constituents of Samples G and H as trimethylsilyl ether derivatives.

Figure reference*	$I^{PLOT\ OV\text{-}1}_{250^oC}$**	M^{+}*** (%)	Base peak	Prominent ions (%)	Suggested structure****
Sample G (minor constituents)					
1	3160	456 (72)	133	351,343,253,191,131,129 (39)(31)(59)(85)(90)(87)	5α-cholesta-7,22-dien-3β-ol
2	3217	470 (10)	133	373,366,355,343,296,255,191,129 (10)(10)(20)(23)(16)(39)(90)(89)	24-methyl-5,22-cholestadien-3β-ol (3170)
3	3221	472	221	191,133,129	
4	3302	486	129	396,357,191,133	24-ethyl-5-cholesten-3β-ol (3305)
Sample H (minor constituents)					
1	3017	442	143	255,341,357,382	
2	3088	456	111	255,191,133,129	5,22-cholestadien-3β-ol (3060)
3	3103	458	231	107,341,345,374,387	
4	3166	456	131	105,253,343,351,366	5α-cholesta-7,22-dien-3β-ol
5	3222	470	105	343,253,318	24-methyl-5,22-cholestadien-3β-ol (3170)
6	3267	470	343	386	
7	3275	472	245	341,343,345	
8	3296	456	129	233	
9	3306	486	129	397,381,367,255	24-ethyl-5-cholesten-3β-ol (3305)
10	3338				
11	3355	486	137	255,107	

* Relevant chromatograms appear in Figure 3.17.
** Indices calculated by a relay technique based on cholestanol TMS ether I = 3145.
*** Mass spectrometer conditions: ion source temperature 270°C and electron energy 70eV.
**** Standard retention indices for the proposed minor constituents (Table 3.5., Knights[351]) recorded in brackets, ion whose origin support these assignments are underlined.

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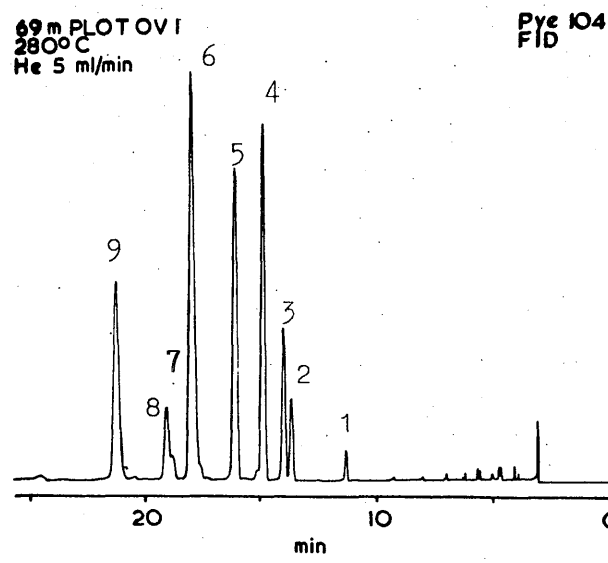


Figure 3.18. Separation of a mixture of fully saturated and side-chain monounsaturated 19-nor-sterols as TMS ether derivatives on a 69m Silanox-type glass open tubular column (PLOT) coated with OV-1. The column was installed in a Pye Model 104 gas chromatograph equipped with FID and operated at 275°C with helium as carrier gas at 5ml/min.

Table 3.13. Kováts retention indices for the fully saturated and side-chain monounsaturated 19-nor-sterol TMS ethers separated in the chromatogram shown in Figure 3.18.

Peak	$I^{275°C}_{OV-1}$	Peak assignment*
1	2909	19,24-bis-norcholest-22-en-3β-ol
2	2991	19-nor-5α-cholest-cis-22-en-3β-ol**
3	3027	19-nor-5α-cholest-trans-22-en-3β-ol
4	3119	19-nor-5α-cholestan-3β-ol
5	3197	19-nor-24-methyl-5α-cholest-trans-22-en-3β-ol
6	3239	19-nor-24-methyl-5α-cholestan-3β-ol
7	3253	19-nor-C_{28}-sterol
8	3258	19-nor-24-ethyl-5α-cholest-trans-22-en-3β-ol
9	3296	19-nor-24-ethyl-5α-cholestan-3β-ol

* Based on gas chromatographic and mass spectral evidence, see Tables 3.14. and 3.15.

** Component previously unobserved.

of Naples. This was shown to be a mixture of 19-nor-sterols which were resolved by t.l.c. and column chromatography (Al_2O_3-$AgNO_3$) and characterised by spectroscopic and classical chemical transformations, the major component, 19-nor-5α,10β-ergost-trans-22-en-3β-ol being isolated and confirmed by independent synthesis.[402]

A sample of this sterol mixture was obtained for evaluation on open tubular glass columns and the chromatogram is shown in Figure 3.18. Kováts retention indices were obtained from co-injected n-alkanes and were of good quality, the condition in the Pye 104 gas chromatograph being well controlled. These are recorded in Table 3.13. The same column installed in the LKB 9000, providing approximately the same resolution (i.e. $R_{2,3} > 1$) afforded the m.s. data summarized in Tables 3.14. and 3.15.

The mass features of these compounds were observed to be very similar to other 5α-stanol and Δ^{22}-sterol TMS ethers and the predominant processes are shown in Figure 3.19 (A and B). The composition of the mixture generally conformed to the observations of Minale and Sodano. In addition to 19-nor-5α-cholest-trans-22-en-3β-ol (peak 3) the cis-isomer was identified - identical in m.s. behaviour and eluted 26 index units earlier. A minor component (peak 7) eluting 5 index units before 19-nor-24-ethyl-5α-cholest-trans-22-en-3β-ol (peak 8) was also observed. This showed an m.s. identical to peak 8 and might be rationalised as the cis-Δ^{22} compound. The cis-trans increment would be expected to be of the order of 20 index units. A minor 19-nor-cholestanol with an unusually constituted C_{10} side-chain was observed by Minale and Sodano[402] and peak 7 may be assigned to this without possible comment on the nature of the side-chain structure. The 19-nor-stanol with the 24(28) methylene C_9 side-chain observed by the original workers was absent from this mixture making it seem likely that the current sample was isolated during an $AgNO_3$ chromatography step intended to obtain this compound. Biosynthetic studies by De Rosa, Minale and Sodano have demonstrated that these sterols arise mainly by modification of dietary sterols and not de novo from acetate.[403]

(d) Saturated sterols from Axinella verrucosa. A mixture of sterols, isolated as a fraction by column chromatography (Al_2O_3-$AgNO_3$) on the acetylated sterols of the Axinella verrucosa, was obtained from the Minale laboratory. The total sterols of this animal collected from the Bay of Naples have been shown to be a unique mixture of 3β-hydroxymethyl-A-nor-5α-cholestanes with conventional C_8, C_9 and C_{10} side-chains.[404] The separation of this stanol fraction as TMS ethers on a glass open tubular column is shown in Figure 3.20, indicating a

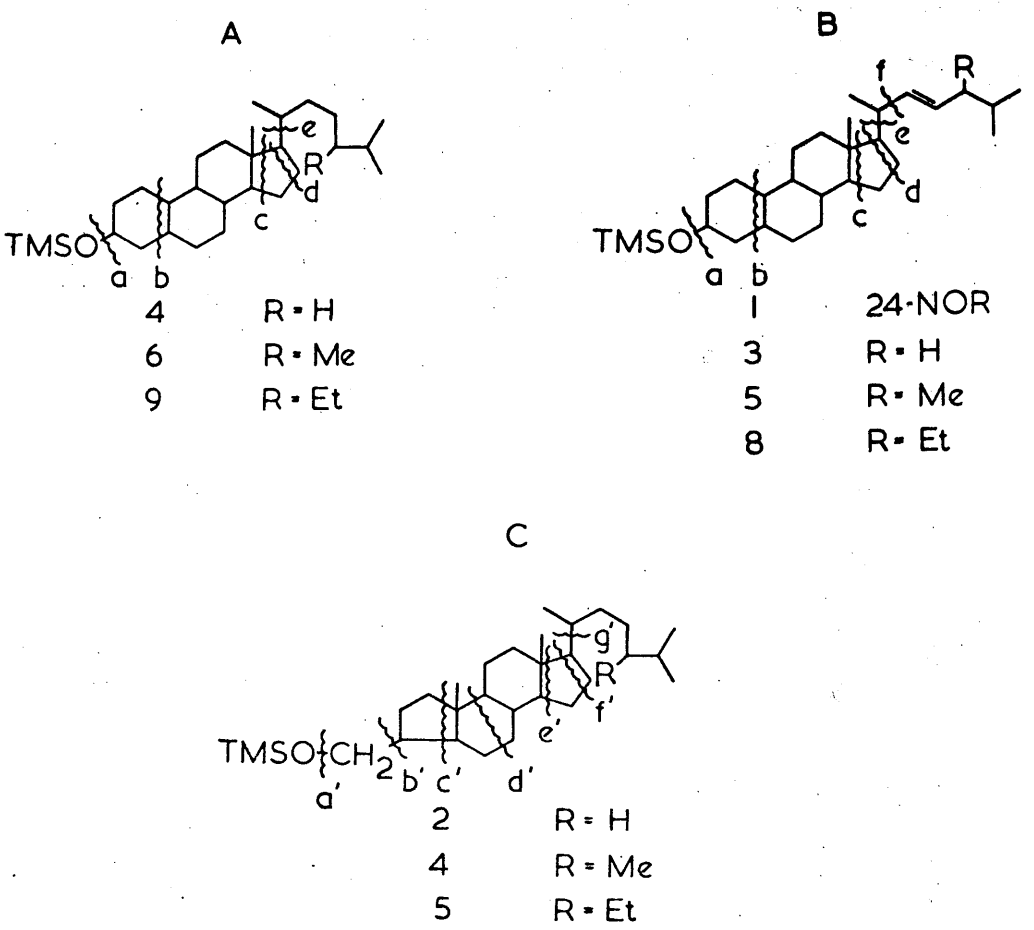


Figure 3.19. Prominent mass spectral fragmentation at 70eV for 19-nor-stanols (A), side-chain unsaturated 19-nor-sterols (B) and saturated A-nor-stanols. The numbered homologues refer to the peaks in chromatograms shown in Figure 3.18.for the 19-nor-sterols and Figure 3.20. for the A-nor-stanols.

Table 3.14. Salient features of the mass spectra of the TMS ether derivatives of the saturated 19-nor-sterols.

Ion	19-Nor-cholestan-3β-ol(4)* MW 446	24-Methyl-19-nor-cholestan-3β-ol(6)* MW 460	24-Ethyl-19-nor-cholestan-3β-ol(9)* MW 474
$M^{+\cdot}$	7	9	9
$[M-15]^{+}$	12	13	10
$[M-90]^{+\cdot}$	85	100	84
$[M-90-15]^{+}$	13	13	11
m/e 201 u	100	100	100
291 v	13	16	15
243 w	26	27	25
216 x	40	35	37
187 y	18	15	16
147 z	30	27	26
121	24	24	26
95	38	32	34

**

u) Base peak, M-(side-chain and C_{15}-C_{17}[c]+90[a]).

v) M-(side-chain and C_{15}-C_{17}[c]+H).

w) M-(side-chain [e]+90[a]).

x) M-(side-chain and C_{15}-C_{16}[d]).

y) M-(side-chain [e]+90[a]+Me).

z) Cleavage of ring A[b].

* Reference by number to peaks in chromatogram shown in Figure 3.22.

** Bracketed letters [ ] refer to Figure 3.23. (section A).

Table 3.15. Salient features of the mass spectra of the TMS ether derivatives of side-chain unsaturated 19-nor-sterols.

Ion	19,24-Bis-norcholest-22-en-3β-ol(1)* MW 430	19-Nor-5α-cholest-22-en-3β-ol(3)* MW 444	19-Nor-5α,10β-ergost-trans-22-en-3 -ol(5)* MW 458	24-Ethyl-19-nor-5α-cholest-22-en-3β-ol(8)* MW 472
M^+	7	5	10	13
$[M-15]^+$	6	4	4	6
$[M-90]^{+\cdot}$	16	17	22	28
$[M-90-15]^+$	19	7	2	6
m/e 243 t	100	100	100	100
360 u	22	27	20	18
331 v	19	17	22	31
270 w	18	17	17	19
215 x	13	9	14	13
201 y	14	19	18	23
147 z	55	52	59	74
121	37	38	47	69

**

t) Base peak, M-(side-chain [e]+90[a]).

u) M-(vinylic cleavage [f]).

v) M-(side-chain [e]+90[a]+2H).

w) M-(vinylic cleavage [f]+90[a]+H).

x) M-(side-chain and C_{16}-C_{17}[d]).

y) M-(side-chain and C_{15}-C_{17}[c]).

z) M-(cleavage at C_1-C_{10} and C_4-C_5 [b]).

* Reference by number to peaks in chromatogram shown in Figure 3.22.

** Bracketed letters [ ] refer to Figure 3.23. (section B).

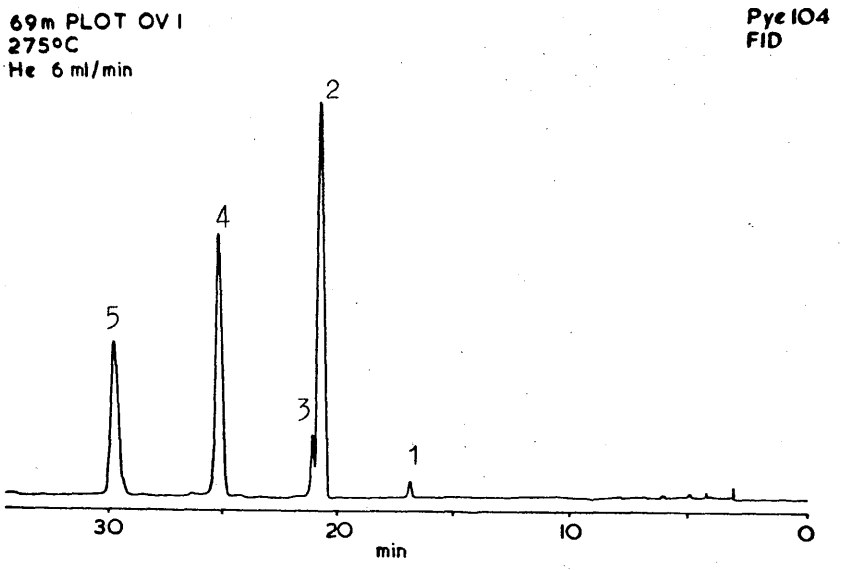


Figure 3.20. Separation of a mixture of fully saturated 3β-hydroxymethyl-A-nor-5α-steranes as TMS ether derivatives on a 69m Silanox-type glass open tubular column (PLOT) coated with OV-1. The column was installed in a Pye Model 104 gas chromatograph equipped with FID and operated isothermally at 275°C with helium as carrier gas at 5ml/min.

Table 3.16. Kováts retention indices for the 3α-hydroxymethyl-A-nor-5α-sterane TMS ethers separated in the chromatogram shown in Figure 3.20.

Peak	$I^{275°C}_{OV-1}$	Peak assignment*
1	3031	3β-hydroxymethyl-A-nor-5α-C_{26}-sterane**
2	3155	3β-hydroxymethyl-A-nor-5α-cholestane
3	3165	3β-hydroxymethyl-A-nor-5α-C_{27}-sterane**
4	3252	3β-hydroxymethyl-A-nor-24-methyl-5α-cholestane
5	3327	3β-hydroxymethyl-A-nor-24-ethyl-5α-cholestane

* Based on gas chromatographic and mass spectral evidence, see Table 3.17.

** Components previously unobserved.

Table 3.17. Salient features of the mass spectra of the TMS ether derivatives of A-nor-stanols.

Ion	3β-Hydroxymethyl A-nor-5α-C_{26}-sterane (1)* MW 446	3β-Hydroxymethyl A-nor-5α-cholestane (2)* MW 460	24-Methyl 3β-hydroxymethyl A-nor-5α-cholestane (4)* MW 474	24-Ethyl 3β-hydroxymethyl A-nor-5α-cholestane (5)* MW 488
$M^+_\bullet$	44	35	32	37
$[M-15]^+$	21	18	16	17
$[M-90]^+_\bullet$	51	47	45	49
$[M-90-15]^+$	29	22	19	19
$[M-103]^+$ } t	26	21	19	20
m/e 103 } t	53	64	60	60
$[M-197]^+$ } u	11	11	10	10
m/e 107 } u	65	73	69	72
215 v	100	100	100	100
201 } w	23	22	23	24
203 } w	55	58	53	53
145 x	35	44	36	37
147	48	59	50	56
257 y	11	8	10	14
306 z	25	24	23	24
121	56	70	68	72

**

t. Cleavage α to TMS moiety and charge retention on nuclear or TMS containing fragment [b]

u. Cleavage across ring B.

v. Base peak, M-(sidechain and C(15)-C(17) [e'] + 90 [a'])

w. M-(α-cleavage to TMS moiety [b'] + sidechain and C(15)-C(17) [e'] with and without transfer of 2H to nuclear fragments)

x. M-(ring A fragment across C(4)-C(5) and C(1)-C(10) [c'] with and without transfer of 2H to nuclear fragment)

y. M-(sidechain [g'] + 90 [a'])

z. M-(sidechain [g'])

* reference by number to peaks in chromatogram shown in Figure 3.24.

** bracketed letters [] refer to Figure 3.23 (section C).

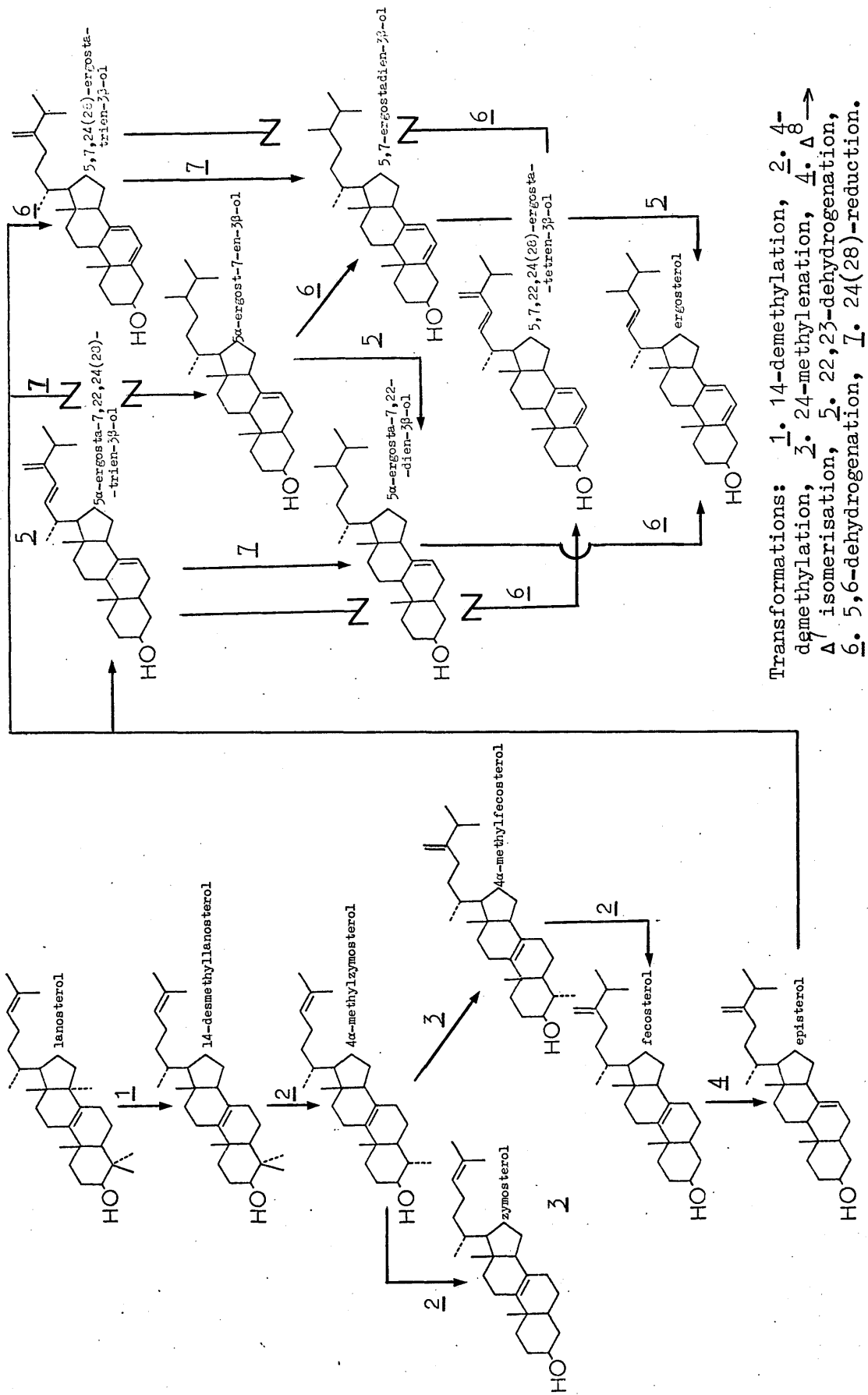
series of four well separated homologues. The retention data, shown in Table 3.16, confirm this quasi-homologous relationship and the mass spectra, summarised in Table 3.17. show molecular weights and base peaks of m/e 215 consistent with saturated C_{29}, C_{28}, C_{27} and C_{26} stanols. This latter compound, a minor component, was not reported by Minale and Sodano.

A prominent distinguishing feature of mass spectra of the 3β-trimethylsilyloxymethyl structure are prominent ions at m/e 103, $(CH_3)_3\overset{+}{Si}O=CH_2$ and $[M-103]^+$. The A-nor configuration of the A-ring influences ring B fragmentation in an unusual way. An ion at $[M-197]^+$ may be rationalised as a cleavage of ring B between C(6)-C(7) and C(9)-C(10) in a fashion familiar in the mass spectra of steroid 4-en-3-ones (485 and references therein). A corresponding ion at m/e 107 arises from transformation of the $[M-90]^+$ ion with charge retention on the ring A fragment. This transition is supported by metastable ion evidence.

An additional minor component (peak 3) partially resolved and eluted 10 index units after 3β-hydroxy-methyl-A-nor-5α-cholestane (peak 2) possessed identical mass spectral features with this compound. The normal ring A structure or a side-chain variation are two possibilities for the structure of this minor constituent. Biosynthetic studies by De Rosa, Minale and Sodano have demonstrated that these sterols arise mainly by modification of dietary sterols and not de nova from acetate.[405]

3.4.2. Yeast sterol mixtures. C_{28} sterols predominate in fungi. Ergosterol is the principal sterol and the other sterols found are principally ergostenol derivatives and can be regarded as ergosterol precursors.[290] The amounts of sterol vary among species but the generally large amounts - ergosterol forms crystalline inclusions in Neurospora crassa[297] - have facilitated biosynthetic studies which involve the isolation of sterols in preparative amounts by a combination of chromatographic methods and the application of a variety of physical and chemical techniques to determine their structures. Notable are the investigations of sterol biosynthesis in various species of yeast (306 and references therein). As the number of identified compounds increases a scheme for the biosynthetic transformations may be devised by considering only single-step transformations of known constituents. Ergosterol is formed from lanosterol which is in turn the biosynthetic product of mevalonic acid

Scheme 3.6. Ergosterol biosynthesis.[407]



Transformations: 1. 14-demethylation, 2. 4-demethylation, 3. 24-methylenation, 4. $\Delta^8 \rightarrow \Delta^7$ isomerisation, 5. 22,23-dehydrogenation, 6. 5,6-dehydrogenation, 7. 24(28)-reduction.

via squalene. Barton and co-workers have proposed such a scheme for ergosterol biosynthesis in Saccharomyces cerevisiae which is shown in Scheme 3.6.[407] A similar scheme has been proposed by Fryberg and co-workers.[408] This scheme proceeds from lanosterol via 14-demethyllanosterol (14-demethylation), 4α-methylzymosterol (4-demethylation) and zymosterol (4-demethylation). 4α-Methylzymosterol may also be incorporated into ergosterol without proceeding via zymosterol indicating a second pathway at this early point in the scheme. 4-Demethylation and 24-methylenation (or vice versa) lead to fecosterol which is transformed to episterol ($\Delta^8 \rightarrow \Delta^7$ isomerization) before the pathways diverge among the large number of intermediates shown in the latter stages of the scheme. The final steps encompass at least three enzyme systems acting for 22,23-dehydrogenation, 5,6-dehydrogenation and reduction of the 24(28) double bond. These systems act on a range of substrates and do so irreversibly under normal growth conditions to yield ergosterol. Thus multiple pathways without apparent unique biosynthetic sequence seem to characterise ergosterol biosynthesis. Among strains of S. cerevisiae the relative proportions of these sterols vary widely, indicating a system of diverse interdependent processes.

Two methods have found general application in the substantiation of these proposed biosynthetic pathways. In feeding experiments appropriately labelled precursors are metabolised in vivo and the transformations are observed by the appearance of the label in subsequent stages of the biosynthetic sequence. Such feeding experiments have been undertaken with results which have confirmed the general validity of the proposed scheme for ergosterol biosynthesis.[407,409] A more specific method involves the isolation of the enzymes or enzyme complex responsible for a specific transformation and the demonstration of its efficacy on the appropriate substrate in cell free systems. This technique has likewise found application in the confirmation of the scheme for ergosterol biosynthesis.[410]

A number of nystatin-resistant mutants of Saccharomyces cerevisiae were observed to differ characteristically in the composition of their sterols. These have provided an elegant means of testing the proposed scheme. Each mutant lacks, wholly or partially, the enzyme involved in a particular biosynthetic step. The effect in each of the four mutants studied is to one of the steps: introduction of the 24-methyl group, the introduction of the 5,6- or 22,23-double bond , or the

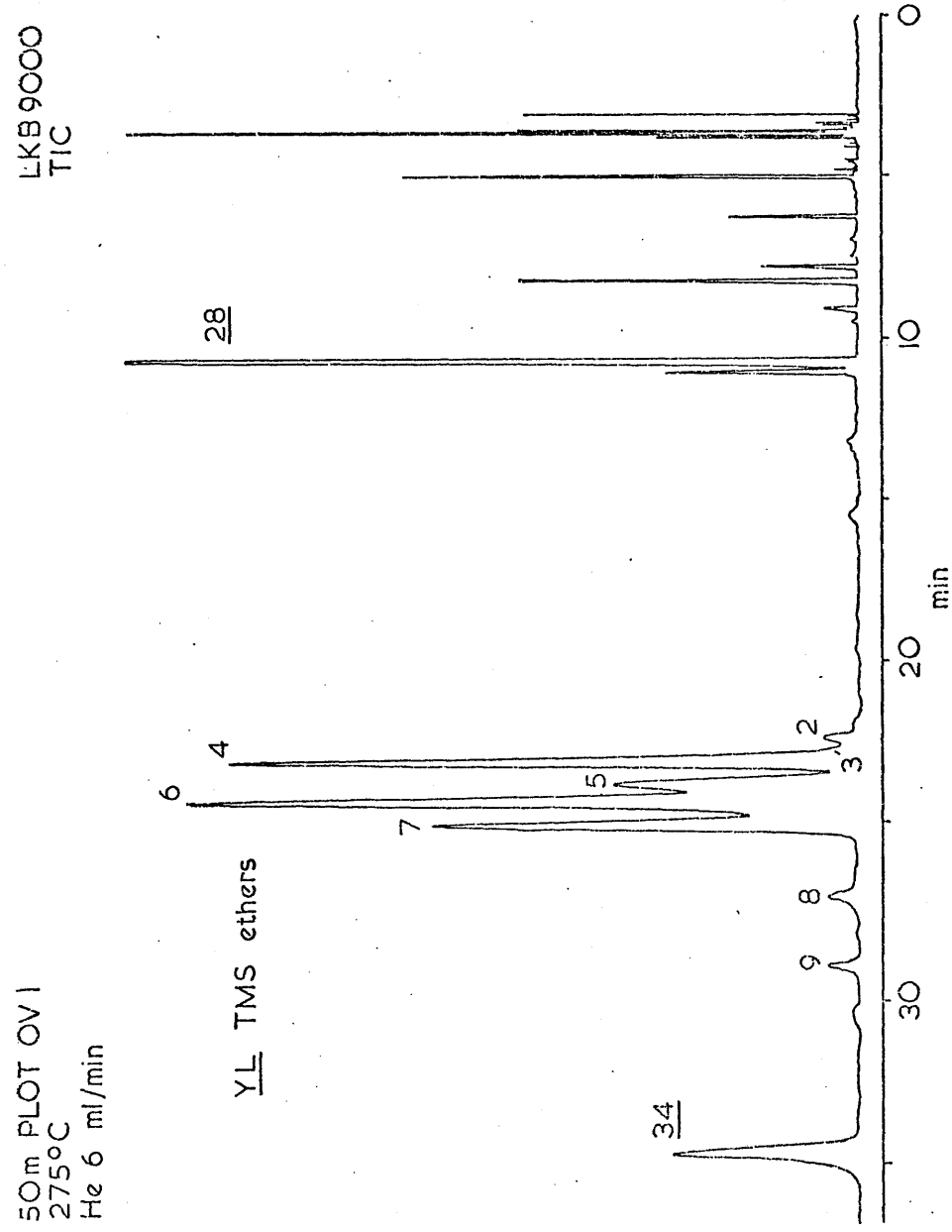


Figure 3.22. Total ionisation current (TIC) chromatogram for the separation of the sterols of mutant YL of the yeast Candida albicans on a 50m Silanox-type (PLOT) glass OV-1 column. Column was installed in the LKB 9000, helium flow rate 6ml/min, column temperature 275°C, source temperature 270°C, electron energy 70eV.

$\Delta^8 \rightarrow \Delta^7$ isomerisation. This is demonstrated by the accumulation of an array of precursors in front of the block in the scheme. These sterol mixtures have been analysed by preparative methods and each isolated component has been characterised by physical and chemical techniques.[411] The results have confirmed the proposed scheme and in some cases the blocks have served to enhance the concentrations of important intermediates which might not have been observed. Investigations of a series of doubly mutated strains, in which two biosynthetic transformations are inhibited, have likewise demonstrated the validity of the Barton scheme.[412]

Sterols of two mutants of Candida albicans.

In the period 1969-1971 work was undertaken in this laboratory on the analysis of yeast sterol mixtures obtained from the laboratory of Professor Sir Derek Barton, Imperial College of Science and Technology, University of London.[371,413] Some useful results were obtained but the work was hampered by the complexity of these mixtures and the limited resolving power of the packed columns available at that time. The opportunity to apply the higher resolving power of glass open-tubular columns to the g.c.-m.s. analysis of such mixtures arose when mixtures of sterols of the wild type and two mutants of the yeast Candida albicans were obtained from the same laboratory.

(a) G.c. and g.c.-m.s. of XL and YL sterols as TMS ethers. Wild type and two mutant strains of C. albicans, XL and YL, were grown, saponified and extracted by conventional methods[406] and the total sterol mixture (2-3mg) was sent for analysis. Trimethylsilyl ether derivatives were prepared and g.c. and g.c.-m.s. data obtained according to the methods previously described. Figures 3.21. and 3.22. show the separations obtained on a 50m Silanox-type glass open tubular column at 275°C installed in the LKB 9000. A somewhat better performance was obtained on the same column installed in the Pye 104 gas chromatograph at 265°C. The retention indices and mass spectral data are summarised in Tables 3.18. and 3.19. The sterols of the wild type yeast were treated in the same way but the sample proved to be so badly contaminated with non-steroidal materials as to make even the most preliminary examination impossible.

(b) Structural assignments and discussion. The sterol types of XL and YL as TMS ethers are summarised in Table 3.20. together with structurally significant ions, and the components common to the two mixtures are indicated by the partial chromatograms in Figure 3.23. These results indicate that the mixtures consist of eleven separate C_{27},

Table 3.18. Retention indices and salient mass spectral features*** of the sterols of mutant XL as TMS ether derivatives.

Peak reference	Retention index a/b**	$M^{\ddagger}$ (%)	Base peak****	Other ions (%)	Comments
2	3175/3168	452 (25)	362	69 (400) 73 (300) 75 (250) 83 (150) 131 (45) 157 (67) 196 (69) 209 (89) 251 (89) 347 (58) 249 (35)	weak
3	3185/3178	454 (43)	349	69 (260) 73 (210) 75 (180) 91 (42) 109 (39) 119 (51) 143 (89) 197 (45) 251 (44) 323 (43) 439 (10) 253 (10) 131 (40)	moderate
4	3193/-	452 and 456 (25) (50)	109	69 (90) 119 (32) 131 (30) 143 (31) 147 (33) 213 (27) 251 (29) 253 (22) 362 (13) 366 (6) 441 (24) 347 (40)	moderate
5	3201/3192	456 (95)	109	69 (165) 119 (40) 131 (41) 147 (43) 213 (47) 229 (34) 251 (30) 351 (30) 416 (44)	strong
6	3215/3207	454	349	69 (120) 73 (110) 119 (40) 131 (42) 143 (41) 144 (39) 145 (36) 157 (34) 119 (25) 211 (26) 251 (21) 323 (72) 364 (25) 459 (5)	strong
7	3225/3217	468 and 456 (33) (27)	364	69 (210) 73 (220) 81 (93) 109 (82) 119 (69) 131 (68) 143 (62) 253 (51) 337 (57) 343 (98) 378 (25) 441 (33) 454 (11)	moderate
8	3233/3224	452 and 456 (22) (18)	109	69 (66) 73 (81) 119 (30) 131 (27) 145 (22) 213 (16) 239 (12) 251 (19) 265 (4) 280 (11) 321 (4) 343 (44) 347 (48) 362 (8) 229 (6) 129 (20)	strong
9	3241/3231	452 (28)	109	73 (55) 131 (21) 143 (20) 251 (23) 253 (17) 265 (6) 280 (10) 321 (7) 347 (5) 362 (9) 437 (2)	strong
10	3282/3275	470 (69)	365	69 (350) 109 (98) 129 (72) 131 (67) 145 (78) 229 (51) 455 (47)	weak
11	3316/3311	498 (24)	393	69 (180) 73 (140) 109 (68) 129 (68) 483 (51)	weak

* Relevant chromatogram shown in Figure 3.21.
** 50m Silanox-type (PLOT) OV-1 (a) LKB 9000, 275°C, He 6ml/min. (b) Pye 104, 265°C, He 5ml/min.
*** LKB 9000, source temperature 270°C, electron energy 70eV, emission current 60μA.
**** Base peak over m/e 100.

Table 3.19. Retention indices and salient mass spectral features*** of the sterols of mutant YL as TMS ether derivatives.

Peak* reference	Retention index a/b**	M+ (%)	Base peak****	Other ions	Comments
2	3187/3185	454 (68)	349	69 (200) 73 (210) 109 (53) 129 (41) 131 (57) 211 (47) 251 (76) 323 (54) 364 (14)	very weak
3	3196/-	452,456 (20)(90) and 468 (9)	109	69 (130) 73 (130) 129 (39) 131 (36) 145 (50) 157 (49) 251 (47) 253 (30) 347 (48) 351 (21) 363 (22) 454 (22)	weak
4	3203/3198	456 (100)	456	69 (160) 107 (64) 109 (55) 133 (36) 145 (34) 213 (39) 229 (35) 351 (26) 366 (11) 441 (45)	strong
5	3217/3212	454 (44)	349	69 (110) 73 (105) 129 (35) 131 (39) 143 (43) 211 (27) 237 (12) 251 (23) 253 (15) 313 (72) 364 (27)	moderate
6	3228/3223	468 (36)	363	69 (110) 73 (90) 131 (47) 144 (40) 211 (31) 253 (30) 337 (64) 378 (22) 453 (4)	strong
7	3241/3236	452 (22)	109	73 (55) 129 (16) 131 (20) 143 (18) 211 (13) 239 (12) 251 (23) 253 (14) 265 (6) 280 (9) 321 (5) 341 (4) 347 (47) 362 (7)	strong
8	3287/3279	470 (80)	365	69 (190) 73 (200) 75 (200) 129 (60) 131 (80) 227 (33) 339 (76) 380 (37) 455 (22)	weak
9	3317/3315	498 (40)	393	69 (250) 129 (54) 215 (32) 255 (16) 469 (11) 483 (37)	weak

* Relevant chromatogram shown in Figure 3.21.
** 50m Silanox-type (PLOT) OV-1 (a) LKB 9000, 275°C, (b) Pye 104, 265°C, He 5ml/min.
*** LKB 9000, source temperature 270°C, electron energy 70eV, emission current 60μA.
**** Base peak over m/e 100.

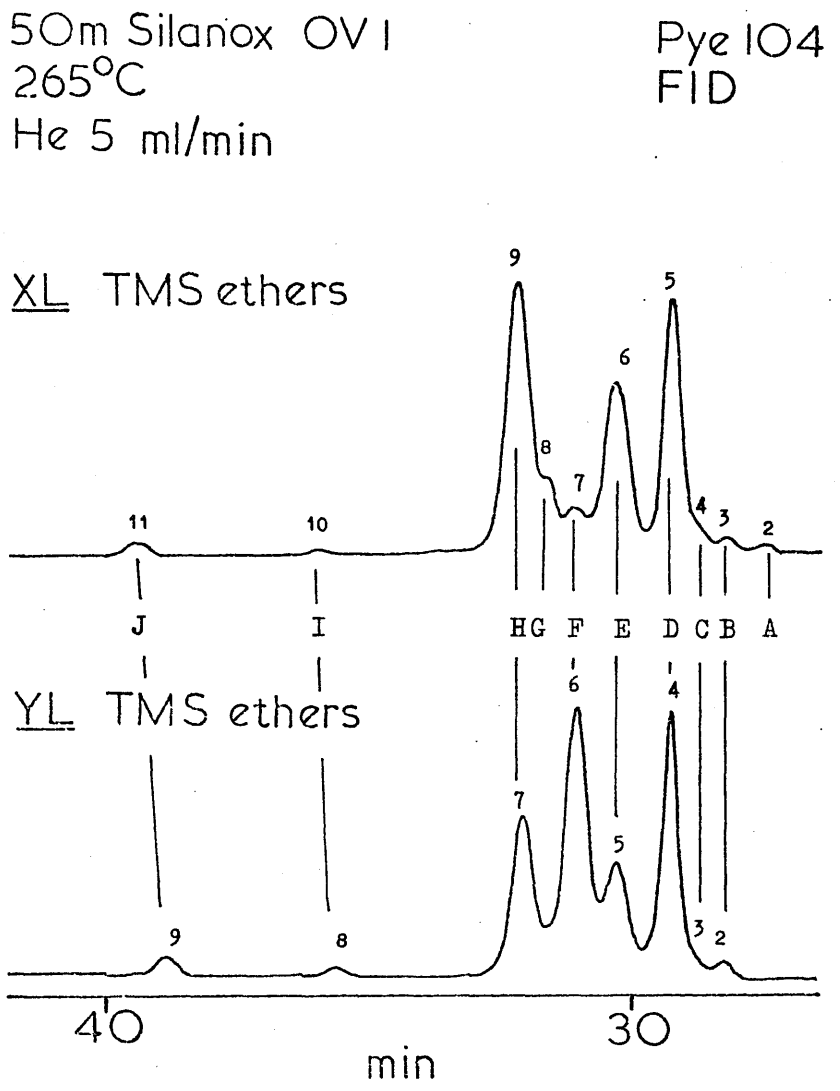


Figure 2.23. Composite chromatograms of the sterols of the mutants XL and YL of the yeast Candida albicans obtained on a 50m Silanox-type (PLOT) glass OV-1 column installed in a Pye 104 gas chromatograph, temperature 265°C, helium carrier gas 5ml/min. Numbers refer to components indexed in Tables 3.18. and 3.19., letters refer to sterol identities summarized in Table 3.20.

Table 3.20. Summary of g.c. and g.c.-m.s. data on Candida albicans mutants XL and YL as TMS ethers.

I*	XL peak	M.+	YL peak	M.+	Base peak	Structurally*** significant ions	Sterol type	Sterol
3171	2	452	-	-	362^{b}	347^{c},$251^{o'}$>$249^{t'}$,$209^{q'}$,69^{v}	C_{27} tetraenol	A
3181	3	454	2	454	349^{c}	439^{a},323^{g},$253^{o'}$<$251^{t'}$,109	C_{27} trienol	B
(approx. 3186)	4	452	3	452	109	362^{b},323^{c},$253^{o'}$>$251^{t'}$	C_{27} tetraenol	C
				468			C_{28} trienol	
		**(456)		(456)				
3195	5	456	4	456	109/456	441^{a},366^{b},351^{c},$253^{t'}$>$251^{o'}$,229,$213^{q'}$	C_{27} dienol	D
3210	6	454	5	454	349^{c}	439^{a},364^{b},323^{g},$253^{o'}$<$251^{t'}$,131^{f},69^{v}	C_{27} trienol	E
3220	7	458	6	468	363^{c}	453^{a},378^{b},337^{g},$253^{o'}$,$211^{q'}$,131^{f}	C_{28} trienol	F
		(456)						
3231	8	456	-	-	109	441^{a},372^{i},343^{t},$255^{o'}$>$253^{t'}$,$213^{q'}$	C_{27} dienol	G
		(452)						
3234	9	452	7	452	109	362^{b},347^{c},341,321^{f},$211^{q'}$,131,129	C_{27} tetraenol	H
3277	10	470	8	470	365^{c}	455^{a}	C_{28} dienol	I
3315	11	498	9	498	393^{c}	483^{a},129^{d},69^{v}	C_{30} dienol	J

* Mean I value from g.c. determination of both mixtures, see Table 3.18 and 3.19.
** Arrows indicate prominent ions arising from incomplete g.c. resolution.
*** Structurally significant ions indexed to Table 3.9.

C_{28} and C_{30}-sterols, nine of which elute in the narrow retention index range of 3170 to 3235. The most clear cut results are the near absence of C_{28}-sterols from XL; the presence of zymosterol (D) in both samples; the presence of ergosterol (F) as a major component in YL and the presence of the trienol (E) and the tetraenol (D) as major components in both samples. A discussion of the assignments, in some cases only partial, based on the g.c. and m.s. data follows.

Sterol A. A C_{27}-tetraenol (MW452) appeared as an early eluting minor component in XL only at I = 3171. This provided a very weak mass spectrum which afforded prominent ions at m/e 209 (frag. q') and m/e 131 (frag. f) indicating a $\Delta^{5,7,x}$ tri-unsaturated nucleus (rings A, B and C). The presence of m/e 251 (frag. o') and m/e 249 at a substantially lower abundance suggest a Δ^{22} side-chain. However, this is inconsistent with the long retention time, 5,7,9(11),24-cholestatetraen-3β-ol providing a more reasonable correlation (24-methyl-5,7,9(11),22-cholestatetraen-3β-ol TMS ether I = 3183 (Table 3.4.); dI loss of 24-methyl group = -100 index units, dI $\Delta^{22} \rightarrow \Delta^{24}$ = +65 index units (Table A.2.), $I_{calc.}$ = 3163) and rationale for the prominence of m/e 69 (frag. v) and m/e 83.

Sterol B. Both mixtures contain a C_{27}-trienol (MW454) as an early eluting minor constituent at I = 3181. The loss of a methyl group and trimethylsilanol (frag. c) forms the base peak and the side-chain cleavage m/e 253 (frag. o') > m/e 251 (frag. t') normally indicates a Δ^{24} structure. Ions at m/e 131 (frag. e) and m/e 323 (frag. f) are characteristic of the 5,7 double bond. However, the occurrence of similar fragments in another C_{27}-trienol (E) jeopordises the assignment of the $\Delta^{5,7,24}$ structure. This is reinforced by poor g.c. correlation: taking zymosterol (D) as reference (I = 3195); dI $\Delta^{8(9)} \rightarrow \Delta^{5,7}$ = +16 index units (Table A.2.), $I_{calc.}$ = 3211. Similar calculations for the Δ^{22} side-chain isomer yield a low value ($I_{calc.}$ = 3130) suggesting an intermediate double bond position in the side-chain as a possibility.

Sterol C. Both mixtures contain a C_{27}-tetraenol (MW452) (I = approx. 3186) almost wholly unresolved from the major zymosterol (D) peak. By consideration of the mixed spectrum it was possible to observe characteristic ions substantiating the molecular weight, m/e 362 (frag. b), m/e 347 (frag. c). The nature and distribution of the double bonds in the nucleus and side-chain are uncertain. The ion at m/e 109 is normally indicative of the $\Delta^{22,24}$ side-chain. However, such a structure leads to a marked retention index decrease and it is impossible to propose a nuclear diene structure which would supply a sufficient compensating effect.

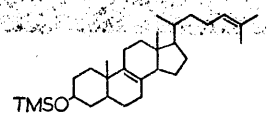
In the YL mixture another C_{28}-trienol (468) appears as an unresolved minor constituent. Likewise, the spectrum of the ternary mixture does not allow structural features of this compound to be reliably distinguished.

Sterol D. The retention behaviour (I = 3195) for this C_{27}-dienol (MW456) agrees with that of authentic zymosterol TMS ether (I^{265C}_{OV-1} = 3189). The mass spectrum of the authentic compound, summarised below, and that of sterol D were effectively identical.

5α-cholesta-8(9),24-dien-3β-ol

456 (100) 69 (400) 75 (74) 107 (56) 81 (42) 213 (39) 93 (34)

105 (34) 229 (31) 351 (30) 367 (12) 255 (12) 227 (10) 343 (10)



$C_{30}H_{52}OSi$
(456)

The $\Delta^{7,24}$ isomer, a known yeast sterol[381,415,416] and the $\Delta^{8(14),24}$ isomer might also be considered on the basis on mass spectra alone. However, with the good correlation of g.c. data and the retention increments for other possibilities (dI $\Delta^{8(9)} \rightarrow \Delta^{8(14)}$ = -23, dI $\Delta^{8(9)} \rightarrow \Delta^{7}$ = +25, Table A.2.) being large compared to the errors observed, this assignment seems secure.

Sterol E. Both mixtures contain this C_{27}-trienol (MW454) of I = 3210 The mass spectrum contains a base peak of m/e 349 (frag. c) and ions at m/e 131 (frag. f) and m/e 323 (frag. g) which are characteristic of the 5,7-dienyl TMS ethers. The side-chain fragments m/e 251 (frag. o') m/e 253 (frag. t') indicate a Δ^{24} unsaturation. The sterol 5,7,24-cholestatrien-3β-ol is a known yeast sterol[415,416], but on the basis of m.s. alone the $\Delta^{5,8(9),24}$ is not ruled out. However, retention increments based on zymosterol (I = 3195, dI $\Delta^{8(9)} \rightarrow \Delta^{5,7}$ = +16 index units, Table A.2.) give a calculated retention index of 3211, in good agreement with the experimental value.

Sterol F. The authentic retention index of ergosterol ($I^{265°C}_{OV-1}$ = 3223 and the mass spectrum (Table 3.8. and Appendix B) agree closely with this minor constituent of XL and prominent constituent of YL at I = 3220.

Sterol G. This C_{27}-dienol (MW456) I = 3231 appears as a minor constituent, poorly resolved from the C_{27}-tetraenol (H), in the XL mixture only. The ions at m/e 213 and m/e 229 are consistent with a Δ^{7} structure. Ions of m/e 343 (frag. t), m/e 255 (frag. o'), m/e (frag. t') and m/e 372 (frag. i) are consistent with a side-chain double bond. The high retention index of this C_{27} sterol might suggest the combination of $\Delta^{7,24}$.

Sterol H. This C_{27}-tetraenol (MW452) at I = 3234 is a major constituent in both XL and YL mixtures. Prominent ions at m/e 362 (frag. b) and m/e 341 (frag. c) reinforce the assignment of molecular weight. The base peak at m/e 109 is normally characteristic of the $\Delta^{22,24}$ side-chain diene system. An ion at m/e 341 (frag. t) is also consistent with a diunsaturated C_8-side-chain. A fragment at m/e 211 (frag. q') indicates a diunsaturated nucleus and ions at m/e 321 (frag. g) and m/e 131 (frag. f) might suggest the $\Delta^{5,7}$ system. The retention index (I = 3234) would, however, be inconsistent with the suggestion that sterol H is the Δ^{22} derivative of sterol E: dI $\Delta^{24} \rightarrow \Delta^{22,24}$ is -53 for the cholestane skeleton and -36 for the 24-ethyl analogue (Table A.2.). On the basis of the currently available reference data is it not possible to resolve this inconsistency.

Sterol I. A C_{28}-dienol (MW470) at I = 3277 is a late eluting minor component in both mixtures. Ions at m/e 265 and m/e 227 in the weak spectrum are consistent with a nuclear methylated sterol and this with the retention index (zymosterol I = 3189, dI for 4α-methyl = +54 (Table A.2.) $I_{calc.}$ = 3243) 4α-methyl-zymosterol is a possibility. The 4α-methyl $\Delta^{7,24}$ might also be considered with (dI $\Delta^{8(9)} \rightarrow \Delta^{7}$ = +26 index units) $I_{calc.}$ = 3269.

Sterol J. The final steroidal component in both mixtures is a C_{30}-dienol (MW498). The weak spectrum has common features with standard lanosterol TMS ether[367] and the retention is in reasonable agreement.

(c) Conclusions. This brief investigation, though somewhat inconclusive, demonstrates the utility of glass open-tubular columns in the analysis of complex mixtures of sterols.

The sterols of the wild strain of Candida albicans were not examined but other workers have observed zymosterol and ergosterol in isolable amounts in the wild strain in Saccharomyces cerevisiae. Other minor sterols with side-chain unsaturated systems were also observed by U.V. spectroscopy.[411]

The assignments, with the exception of those for zymosterol and ergosterol, are unsupported by data on authentic compounds. Until further reference data are obtained these must remain tentative. The sterols of yeast so far isolated and characterised are numerous (e.g. 415,406 and references therein). Gas chromatographic and m.s. examination of these would be a productive area for the correlation

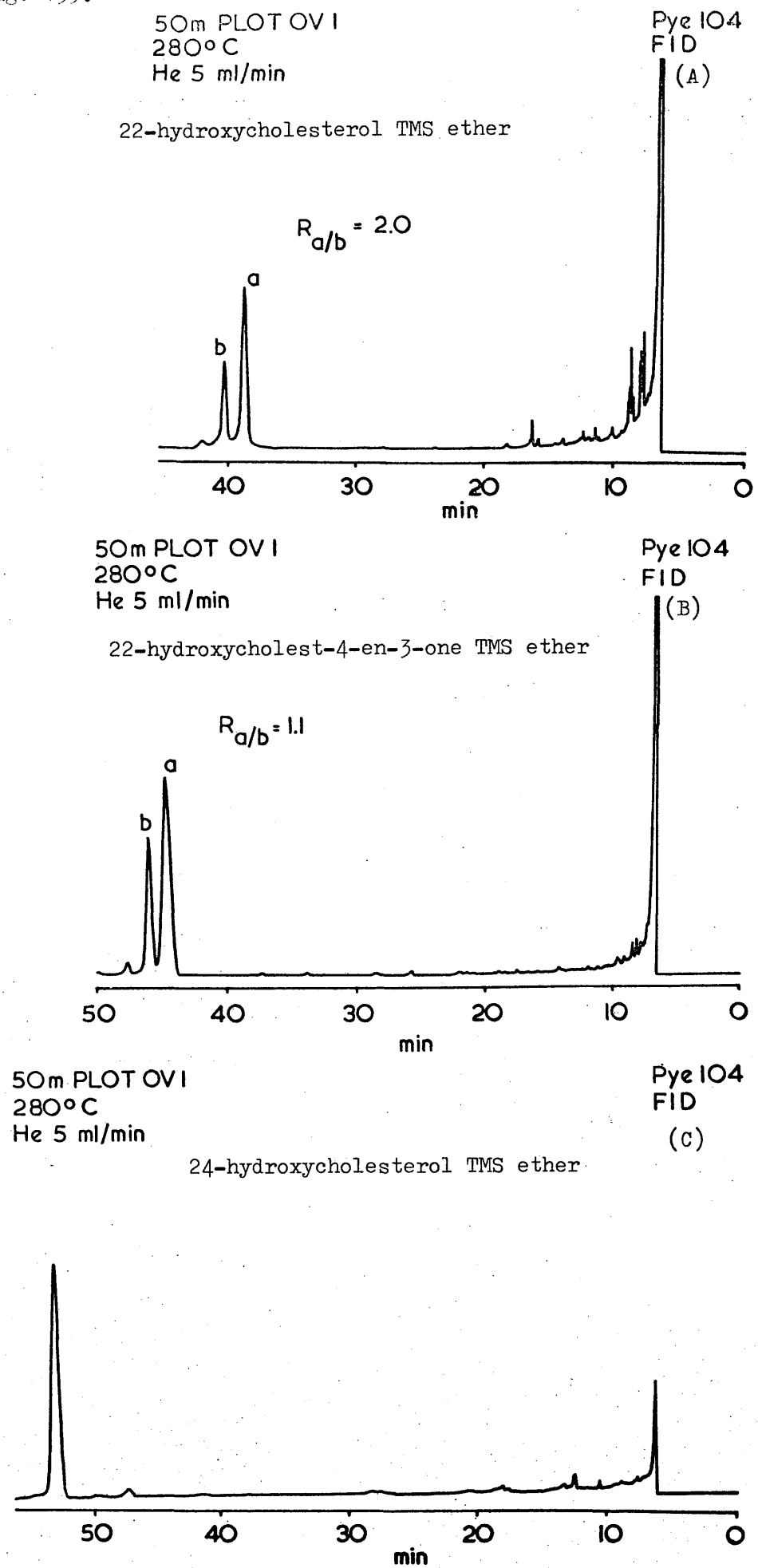


Figure 3.24. Chromatograms of three side-chain hydroxylated steroids as TMS ethers. Complete figure legend appears on the opposite page.

of g.c. and m.s. behaviour with sterol structure, particularly in respect of polyunsaturation.

3.4.3. Miscellaneous applications.

(a) 22(R,S)- and 24(R,S)-side-chain hydroxylated steroid TMS ethers. During the g.c.-m.s. investigation of a long range trimethylsilyl group migration in the electron impact mass spectra of some side-chain hydroxylated Δ^4-3-ketosteroids by Gaskell, Smith and Brooks[417] it was of interest to attempt the resolution of two side-chain epimer structures as their TMS ethers. These were (22R,S)-22-hydroxycholesterol, the corresponding 4-en-3-one and (24R,S)-24-hydroxycholesterol. The chromatograms of these compounds as their TMS ethers are shown in Figure 3.24 and their retention indices are recorded in Table 3.21.

The 22-hydroxy compounds showed a complete or nearly complete resolution, Figure 3.23. (A) and (B). This is not unexpected since packed column gas chromatography showed evidence of separation and 22-hydroxy sterols are resolved by other chromatographic methods.[418] The (24R,S)-compound is unresolved under these conditions. This is consistent with the behaviour so far encountered with other 24-epimeric compounds. It is interesting to note the resolution of 24-epimeric hydroxysteroids as benzoate derivatives by extended irrigation silica gel t.l.c.[419] and by high performance liquid chromatography on a silica gel column.[420]

The mass spectra of these compounds obtained by g.c.-m.s. on the same column are summarised in Table 3.22. These were as expected.[417] However, it is interesting to note the mass spectra of the resolved 22-hydroxycholest-4-en-3-one TMS ether, Figure 3.25. The base peak in both cases is m/e 173, arising from side-chain cleavage α to the trimethylsilyloxy moiety with charge retention on the side-chain fragment, and both show a molecular ion of very low abundance. The ions arising from the long range rearrangement, the side-chain trimethylsilyl group in combination with a fragment of m/e 123 produced by cleavage across ring B, shows slightly differing abundances: m/e 196, first eluted epimer (A) 56%. 7.5% total ionisation; second eluted

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Figure 3.24 (opposite). Open tubular chromatograms of the separation of side-chain epimers (22R, and 22S) of 22-hydroxycholesterol (A) and 22-hydroxycholest-4-en-3-one (B) as the bis- and mono-trimethylsilyl ether derivatives, respectively. Chromatogram (C) is of the unresolved 24-hydroxycholesterol trimethylsilyl ether derivatives.

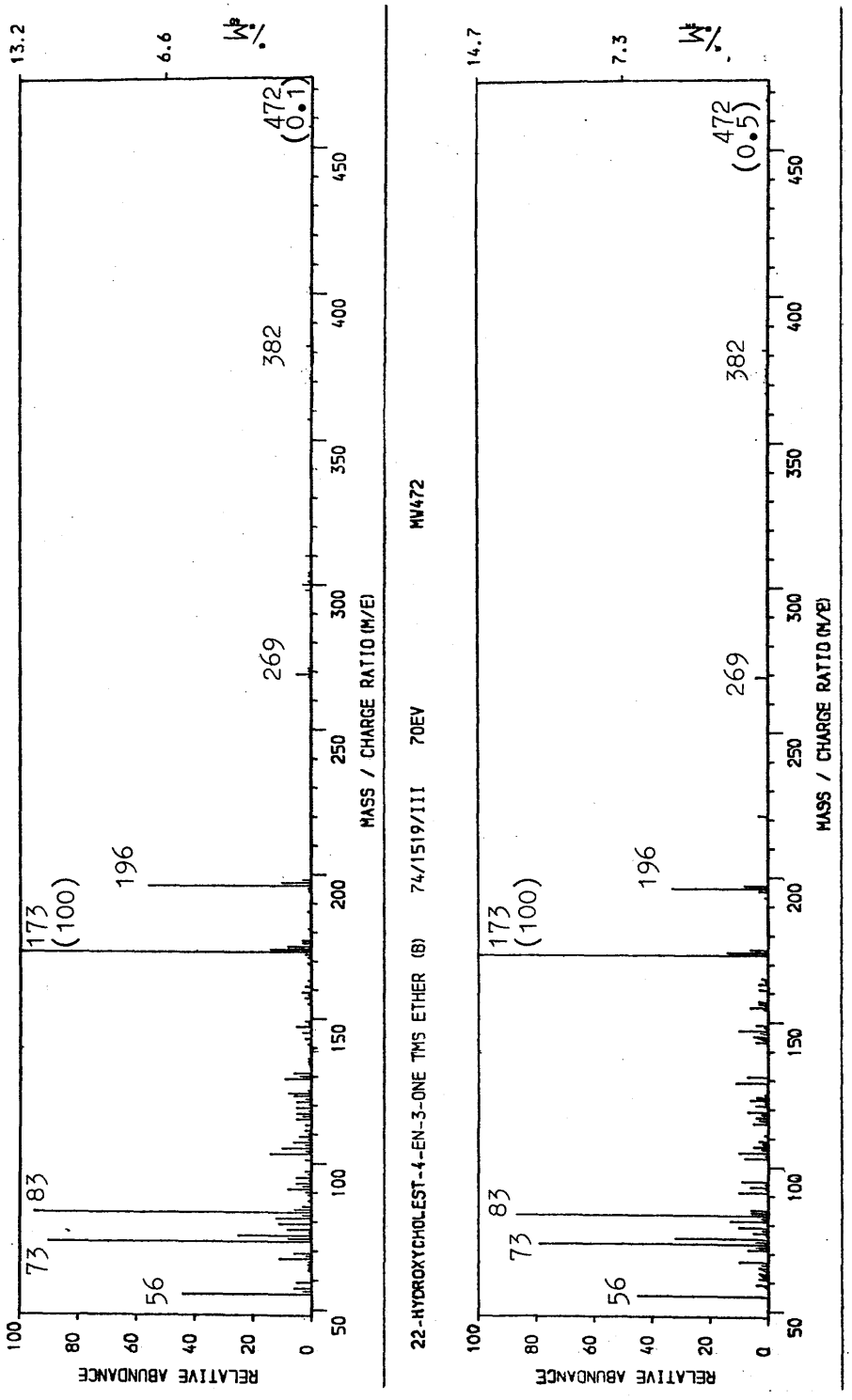

Figure 3.25. Mass spectra obtained on the first eluting (A) and second eluting (B) epimers of 22-hydroxycholest-4-en-3-one trimethylsilyl ether derivatives. These were obtained during g.c.-m.s. analysis on the LKB 9000 equipped with a 50m PLOT OV-1 open tubular column at 275°C with helium as carrier gas at 6ml/min. Source temperature was 270°C and electron energy 70eV.

Table 3.22. Salient features of the mass spectra of three side-chain hydroxylated steroids as trimethylsilyl ethers.

Compound	$M^{+\cdot}$	base peak	M-15 $^{+}$	M-90 $^{+\cdot}$	M-105 $^{+}$	α-cleavage*** a	α-cleavage*** b	Other significant ions
22-Hydroxycholesterol								
	546	173	531	456	441	173	475	417(M-129) 327(M-219) 255 129
(a)*	(0.8)		(0.4)	(0.4)	(0.1)	(100)	(0.5)	(0.1) (0.1) (0.8) (5)
(b)*	(0.8)		(0.2)	(0.1)	(0.2)	(100)	(0.2)	(0.1) (0.1) (0.5) (5)
22-Hydroxycholest-4-en-3-one								
	472	173	457	382	367	173	299	271 269 229 196 124
(a)*	(0.1)		(0.1)	(1)	(0.1)	(100)	(3)	(1) (5) (1) (56) (8)
(b)*	(0.5)		(0.5)	(1)	(0.5)	(100)	(1)	(2) (5) (0.1) (33) (4)
24-Hydroxycholesterol **								
	546	73	531	456	441	143	503	417(M-129) 327(M-219) 255 129
	(2)		(2)	(4)	(2)	(94)	(4)	(1) (7) (5) (58)

* Epimers resolved on 50m PLOT OV-1 open tubular column installed in the LKB 9000 under the same conditions as noted in Figure 3.15. with somewhat lower efficiency; i.e., R ~0.85.

** Mass spectrum gratefully acknowledged, Dr. A.G. Smith, Department of Chemistry, University of Glasgow.

*** α-Cleavage: a = charge retention on side-chain fragment; b = charge retention on fragment including steroid nucleus.

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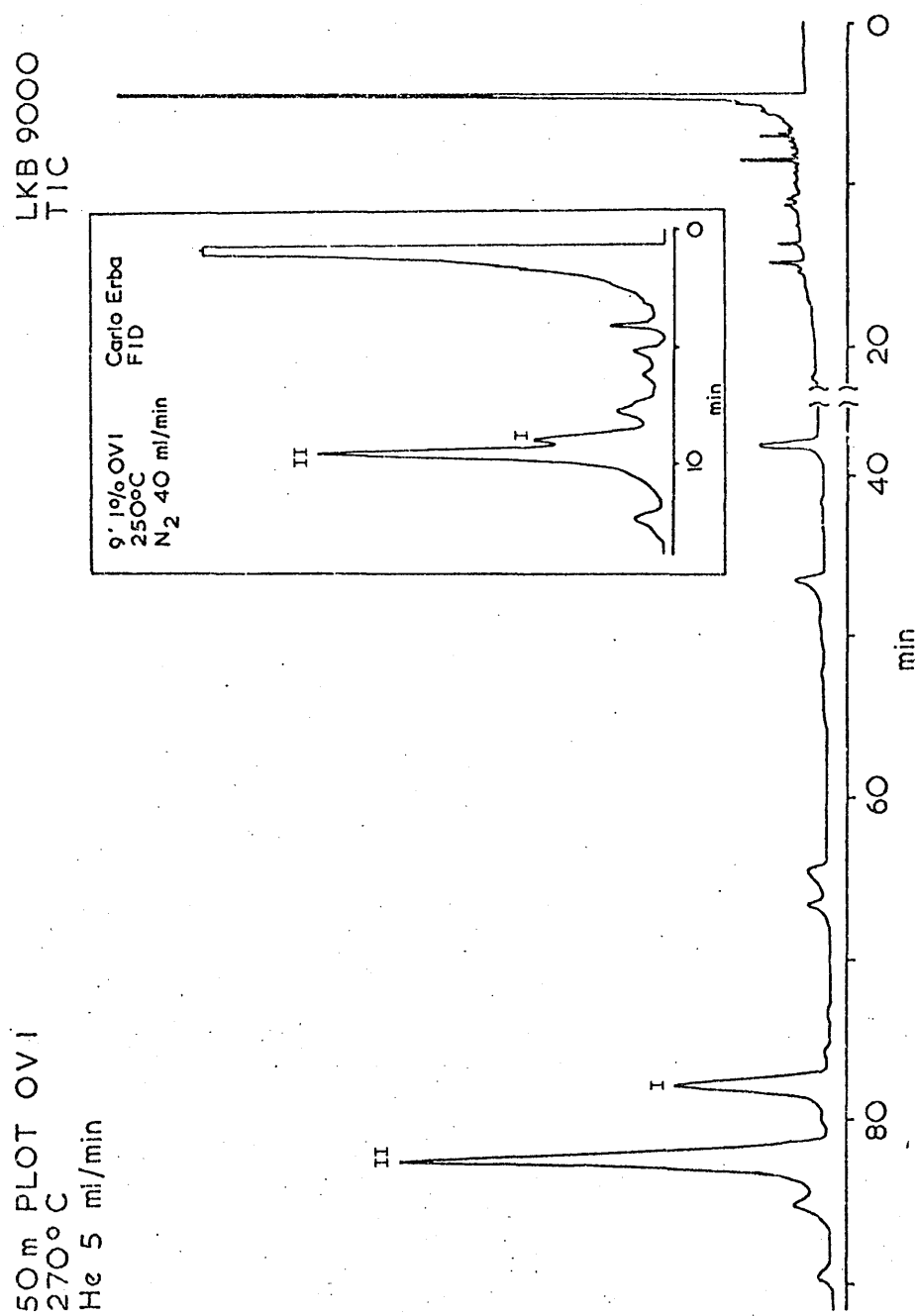

Figure 3.26. Separation of a mixture of sterols obtained from A.R. Bader Company (No. S40467-5) as ergosta-5,8(9),22-trien-3β-ol as their trimethylsilyl ether derivatives on a 50m Silanox-type (PLOT) OV-1 open tubular column installed in the LKB 9000 and on a packed column installed in a Carlo Erba gas chromatograph equipped with a flame ionization detector (insert).

Table 3.21. Kováts retention indices of three side-chain hydroxylated steroids as trimethylsilyl ether derivatives.

Compound	$I^{PLOT\ OV-1*}_{280^{o}}$
22-Hydroxycholesterol	3305 ** 3319
22-Hydroxycholest-4-en-3-one	3362 ** 3383
24-Hydroxycholesterol	3411 **

* Conditions as noted in Figure 3.15.
** Resolved (22R and 22S) epimers.
*** Unresolved (24R and 24S) epimers.

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epimer (B) 33%, 5% total ionisation. This may be attributable to the slightly more facile transfer of the TMS group in one epimeric form.

(b) Characterisation of a sterol sample No. S40467-5 from A.R. Bader. In the accumulation of reference data on C_{28}-sterols outlined earlier a sample designated 5,8(9),22-ergostatrien-3β-ol was obtained from the collection of rare chemicals of A.R. Bader, Aldrich Chemical Co. Ltd., Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL. This was obtained as the acetate derivative and after hydrogenolysis with $LiAlH_4$ and trimethylsilylation of the resulting free sterols, packed column gas chromatography revealed two poorly resolved components and a number of contaminants (insert, Figure 3.26.). The mixture was then subjected to g.c.-m.s. analysis on a 50m Silanox-type (PLOT) glass column installed in the LKB 9000. The TIC chromatogram and mass spectra obtained are shown in Figures 3.26. and 3.27. respectively and the retention data on both packed and open-tubular chromatographic data are recorded in Table 3.23.

The first eluted component (I), I = 3178, was shown to be a C_{28}-trienol (MW468) with a single point of unsaturation in the side-chain (m/e 343, frag. o) located at Δ^{22} by m/e 253 (frag. o') m/e 251 (frag. t'). A strong m/e 69 (frag. v) is also present. 5,8(9),22-Ergostatrien-3β-ol has been identified in the sterol pool of the isolated symbionts of the lichen Xanthoria parietina and the green alga Chlorella ellipsoidae. Lenton and co-workers recorded the mass spectrum of the sterol as the TMS ether. The salient features of this standard spectrum and the present C_{28}-sterol TMS ether are

facing page 142.

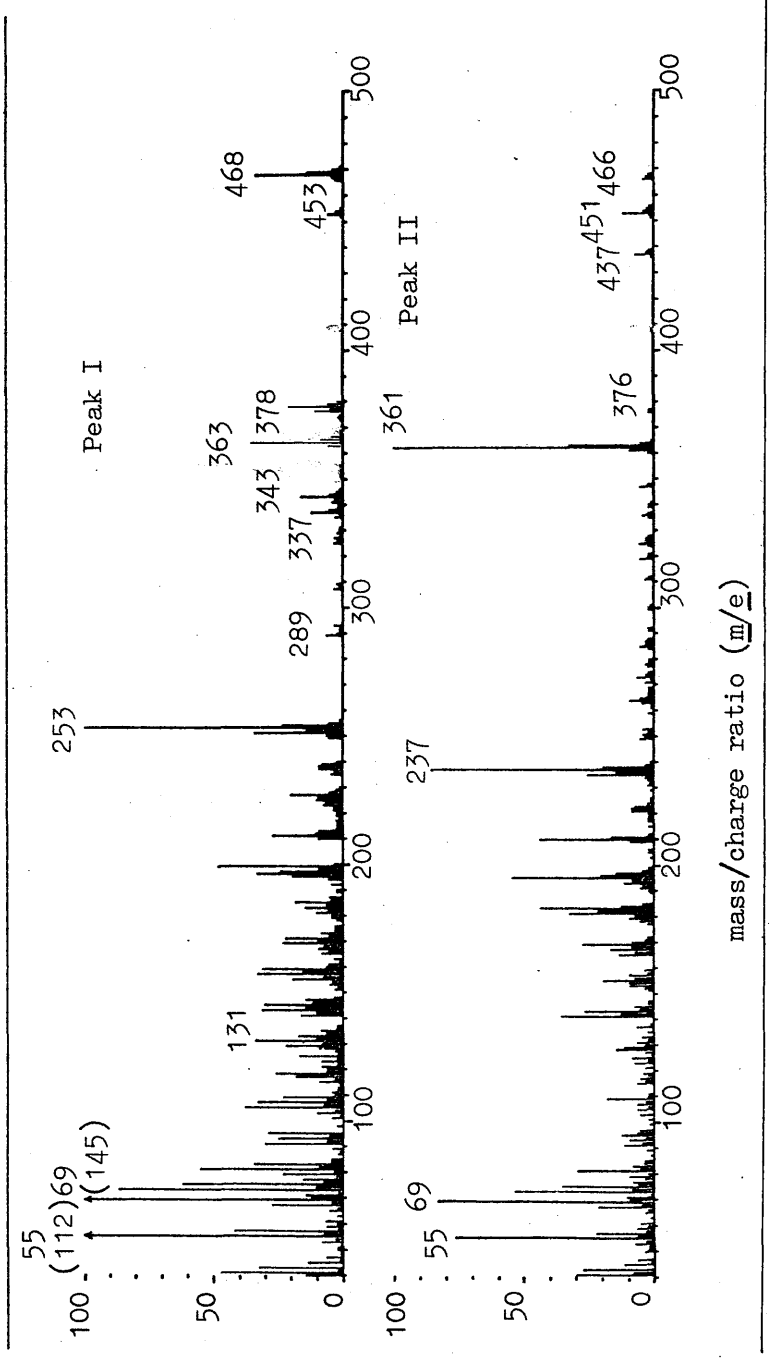


Figure 3.27. Mass spectra of the major sterol constituents of A.R. Bader sample No. S40467-5 sold as ergosta-5,8(9),22-trien-3β-ol as trimethylsilyl ether derivatives. The TIC chromatogram and chromatographic conditions are recorded in Figure 3.25. Source temperature was 270°C and electron energy 70eV.

compared below.

Table 3.23. Kováts retention indices of the major sterol constituents of A.R. Bader Sample No. S40467-5 as trimethylsilyl ethers on packed and open-tubular columns.

Figure reference*	Packed column retention index**	Open-tubular column retention index***
	$I^{OV-1}_{250^oC}$	$I^{PLOT\ OV-1}_{265^oC}$
I	3132	3178
II	3157	3204

* Relevant chromatogram appears in Figure 3.25.

** 9ft. 1% OV-1 Column installed in a Carlo Erba gas chromatograph equipped with flame ionization detector, 250°C, carrier gas nitrogen at 40ml/min. Indices calculated by semi-logarithmic interpolation of co-injected n-alkanes, n-$C_{28}H_{58}$ and n-$C_{34}H_{70}$.

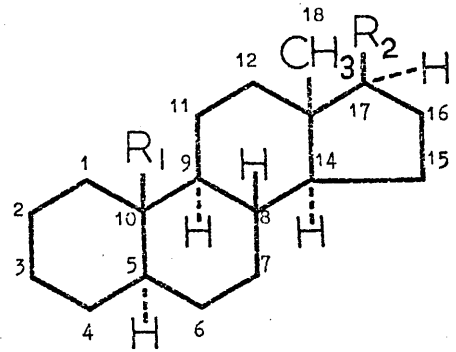
*** 50m PLOT OV-1 open-tubular column installed in a Pye-Unicam Model 104 gas chromatograph equipped with a flame ionization detector. Indices calculated by semi-logarithmic interpolation between appropriate even numbered n-alkanes, co-injected.

ion mass	fragmentation	MS 12/70 eV*	LKB 9000/70 eV
468	$M^{\ddagger}$	67%	34%
453	frag. a	6%	6%
378	frag. b	12%	21%
363	frag. c	100%	36%
337	frag. f	40%	12%
253	frag. o'	25%	100%
211	frag. g'	12%	27%

The data of Lenton were recorded on an AEI MS12 g.c.-m.s. instrument at 70eV* (ion source temperature not recorded). Even allowing for differing instrumental "bias" these data differ substantially, the data of Lenton showing strong similarities to the data recorded for ergosterol itself (Table 3.8.). The retention index of the unknown also tends to undermine the 5,8(9)22-triene assignment. 5,22-Ergostadien-3β-ol $I^{OV-1}_{265^o}$ = 3187, $dI\ \Delta^{5,22} \longrightarrow \Delta^{5,8(9),22}$ = +7 index units (Table A.2.) $I_{calc.}$ = 3194. The retention index of ergosterol differs also.

The second eluted component (II) is a C_{28}-tetraenol (MW466)

with a base peak of m/e 361 (frag. c). There are few other ions of appreciable intensity in the high mass region. An ion of m/e 237 is of uncertain origin and the lack of useful correlations of retention data make the analysis inconclusive. A preparative separation of the two components and examination by u.v. and n.m.r. would be informative.



Parent hydrocarbon	Substitution		Derived class
	R_1	R_2	
5α-oestrane	-H	-H	oestrogens (C_{18} steroids)
5α-androstane	-CH_3 (19)	-H	androgens (C_{19} steroids)
5α-pregnane	-CH_3 (19)	-C(=O)(20)-CH_3 (21)	progestagens (C_{21} steroids)
5α-pregnane	-CH_3 (19)	-C(=O)(20)-CH_2(21)-OH	corticosteroids (C_{21} steroids)*

* Also characterised by a keto or hydroxy group at C(11), and/or a hydroxy group at C(17α)

Table 4.1. Skeletal structure of steroid hormones.

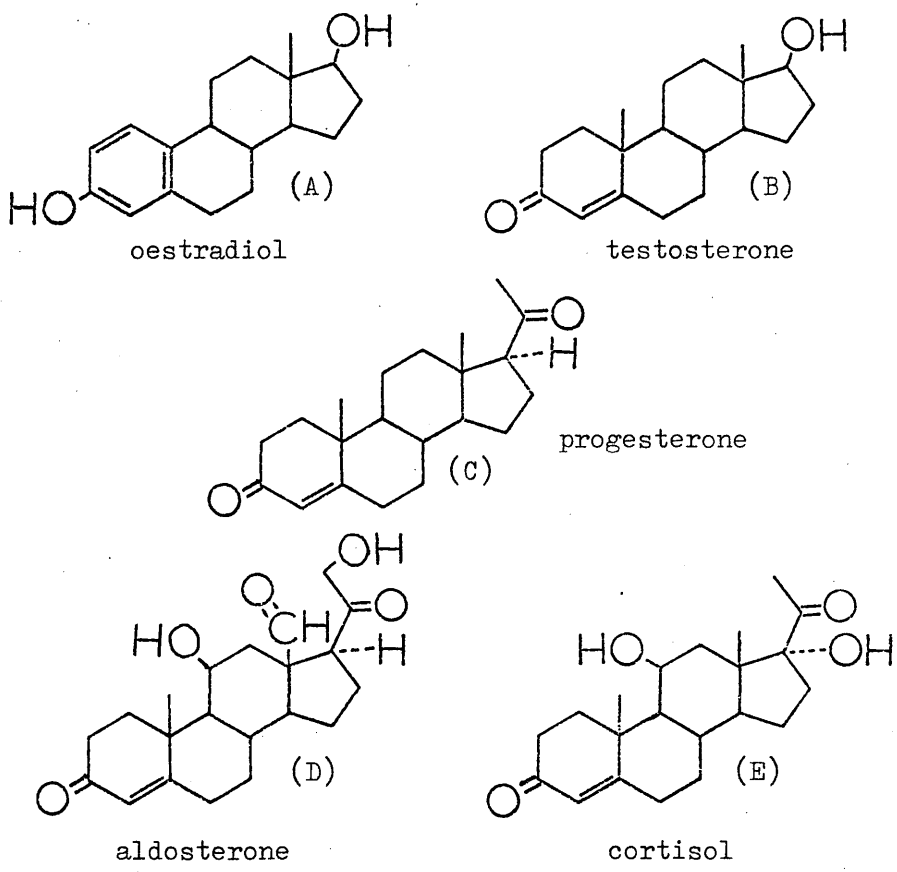


Figure 4.1. Structures of prominent oestrogen (A), androgen (B), progestagen (C), mineralocorticosteroid (D) and glucocorticosteroid (E) hormones.

facing page 144.

4. APPLICATION OF GLASS OPEN TUBULAR CHROMATOGRAPHIC COLUMNS TO THE ANALYSIS OF URINARY STEROIDS.

4.1. Introduction

4.1.1. Structure, classification and nomenclature of steroid hormones.

Hormones are chemical substances, produced by certain cell structures, which when transported to tissue produce a physiological response. Many processes in the human body are regulated by hormones: growth, carbohydrate metabolism, sexual development, electrolyte and nitrogen balance, etc. Several classes of chemical compounds exhibit hormonal activity, individually or in combination (e.g. polypeptides, catecholamines, steroids). The focus of the present study is the steroid hormones. Important hormonally active steroids are secreted by the adrenal gland, the testis, the ovary and the foeto-placental unit. Steroid hormones (henceforth simply steroids) are classified by the number of carbon atoms and are derived from cholesterol, sharing the same perhydro-1,2-cyclopentanophenanthrene structure and nomenclature (Section 3.1.1.). Table 4.1. summarises these structural types and a comprehensive account of the stereochemical features may be found in the textbook by Fieser and Fieser[268] and elsewhere.[269,421]

a) Oestrogens are C_{18} steroids characterised by an aromatic ring A and the absence of the angular methyl group at C(10). The most potent representative of this series is oestradiol (1,3,5(10)-oestratriene-3,17β-diol: Figure 4.1.A). The oestrogens are produced in the ovary, placenta and to a small degree in the adrenal cortex and in combination with other hormones are responsible for the development and maintenance of the female sexual organs.

b) Androgens comprise a group of steroids with a C_{19} structural unit with an angular methyl group at C(10). Testosterone (4-androstene-17β-ol-3-one: (Figure 4.1.B) has the most important androgenic activity in the human body. As a class these regulate sexual development in the male and exhibit anabolic activity - increasing protein synthesis and decreasing amino acid catabolism.

c) Progestagens have a side chain of two carbon atoms at C(17). The main steroid in the progestagen series, progesterone (4-pregnene-3,20-dione: Figure 4.1.C), is produced by the corpus luteum of the ovary and creates a favourable medium for implantation in the uterus of the fertilized ovum. In pregnancy progesterone and the oestrogens are important in the maintenance of foetal well-being.

d) Corticosteroids are manifest by the pregnane nucleus and have a keto or hydroxy group at C(11) and a hydroxy function at C(21). These

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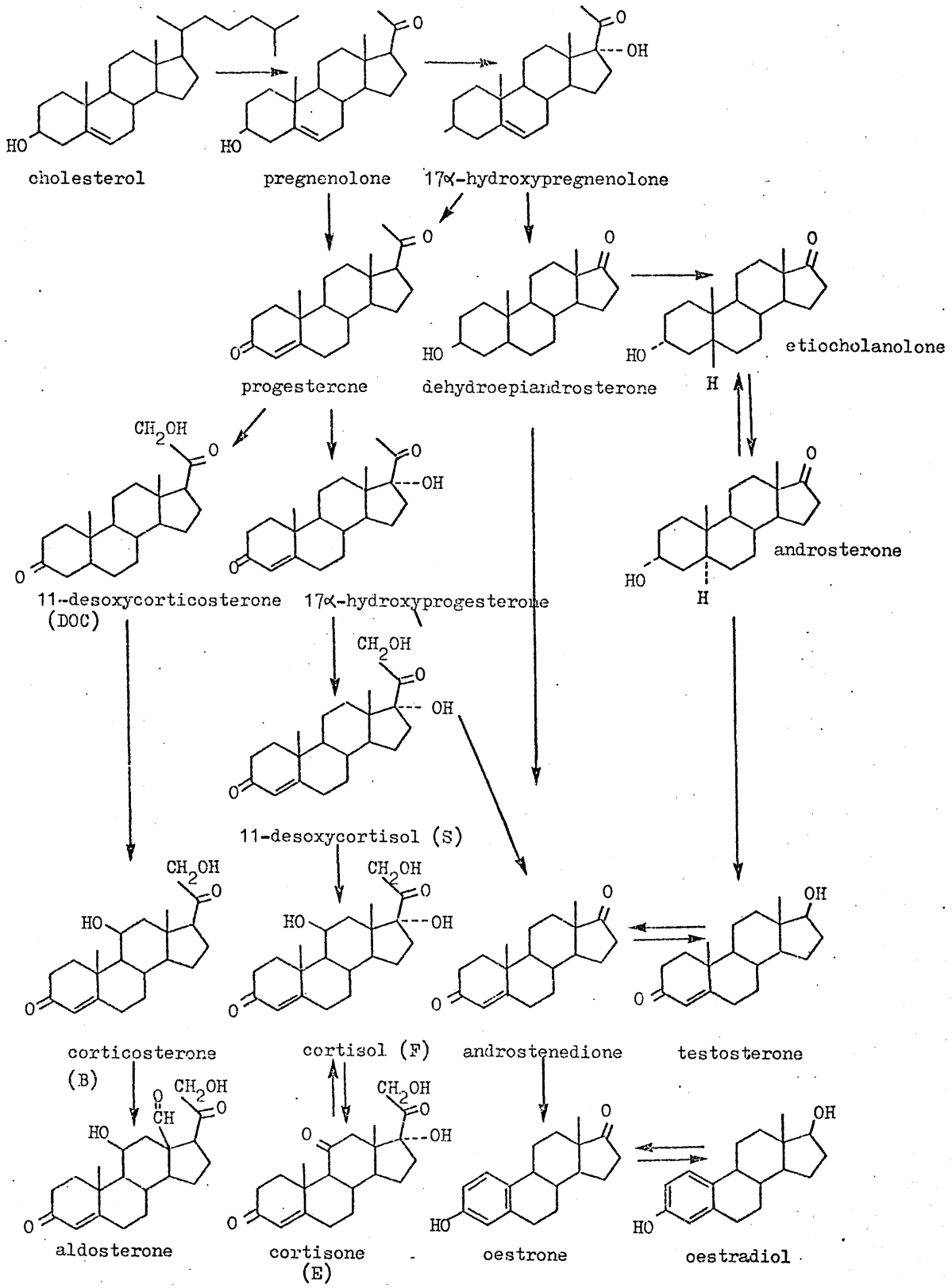


Scheme 4.1. Major pathways of steroid metabolism.

steroids are produced by the adrenals and are divided between the mineralocorticosteroids secreted by the zona glomerulosa and the glucocorticosteroids which originate from the zona fasciculata. Aldosterone (4-pregnene-11β,21-diol-3,20-dione-18-al: Figure 4.2.D) containing an aldehyde at C(18) is the principal mineralocorticosteroid in man. As a group these hormones control salt and water balance, sodium retention and potassium excretion.

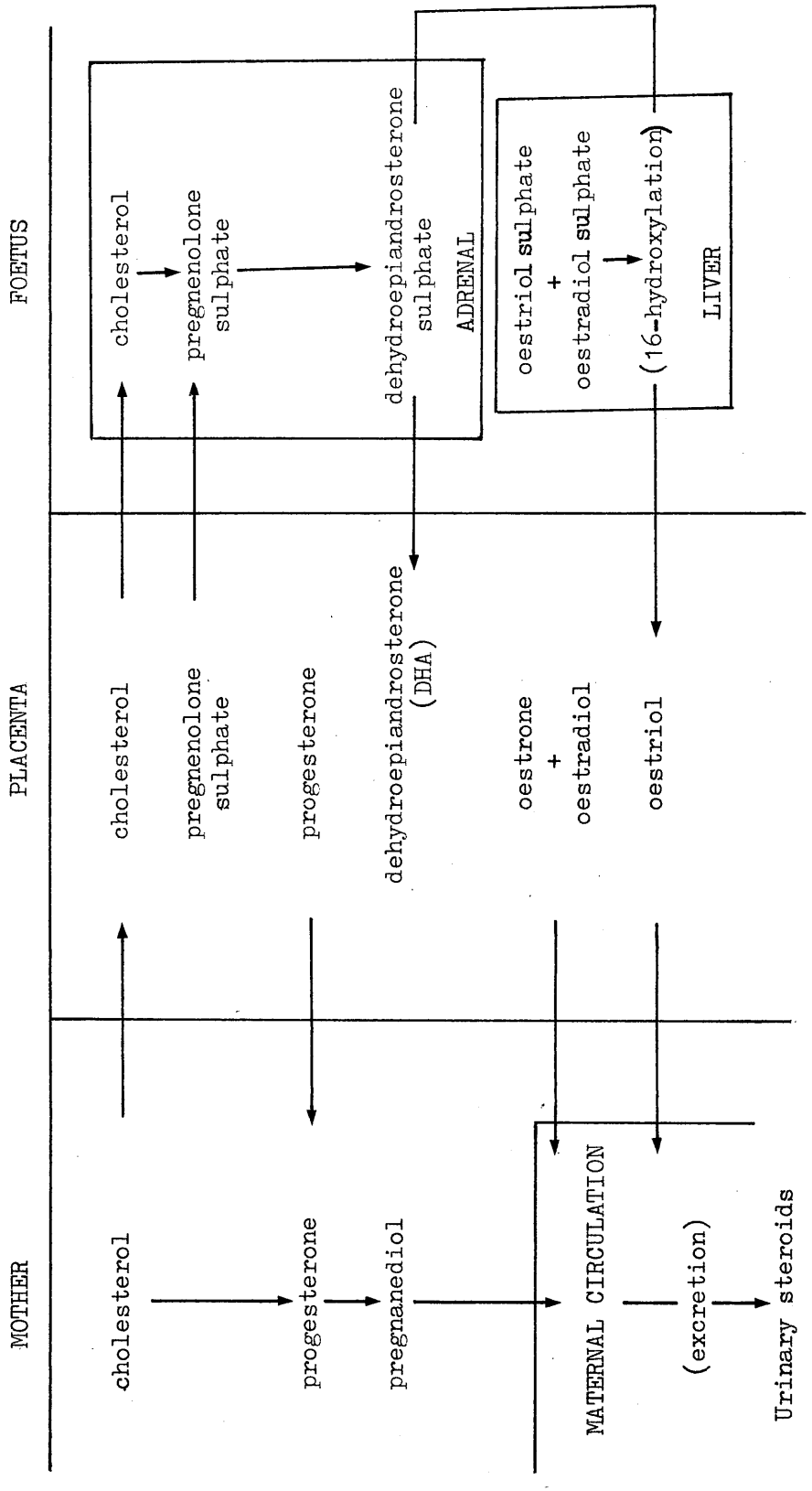
The glucocorticosteroids, such as cortisol (4-pregnene-11α,17β,21-triol-3,20-dione: Figure 4.1.E), stimulate the conversion of protein to carbohydrates and fats by the liver. Other functions are their anti-inflammatory activity, effects on the resistance to shock and infection and effects on membrane permeabilities. These materials may also exhibit a less profound mineralocortico-effect.

4.1.2. Steroid metabolism and the urinary steroids. The synthesis of steroids involves a large number of pathways common to all steroid secreting tissues. The basic steroid nucleus undergoes alterations by enzymes such as hydroxylases, and dehydrogenases, each acting selectively on specific substrates at specific positions. Steroid metabolism has until recently been considered as a straight-forward deactivation mechanism, occurring in the liver and kidneys. This might occur, for example, by conjugation of steroids with glucoronic acid or sulphuric acid. However, evidence for the further metabolism of steroid conjugates indicates a more complex picture.

In urine the bulk of the steroid metabolites are present as water-soluble conjugates with small amounts of free compounds. In blood, all steroids are associated to some extent with plasma proteins; either specifically, as cortisol is coupled to transcortin, or as compounds loosely bound to albumin. Protein interactions with steroid conjugates are also important in the secretion, distribution, and mode of action of steroid hormones. The nature of the receptor proteins and the detailed mechanism by which steroid hormones elicit a physiological response are the object of intensive current investigation.[422]

The analysis of steroids in glandular tissues and the venous effluent of the gland is difficult, particularly in detailed investigations, requiring surgical intervention in sampling. However, hormones and metabolites are continually passed in the urine and this fluid provides a convenient access to these materials. The secretion rates of specific hormones are reflected quantitatively by the concentration of metabolites in urine. Disease states associated with metabolic defects are the hyperfunction, hypofunction or dysfunction

Scheme 4.2. Major pathways of steroid metabolism in the human foeto-placental unit.



of certain endocrine glands.

The effects of treatment may be assessed by analysis of urinary steroids, either individually or in a broad multicomponent "steroid metabolic profile".

Scheme 4.1. shows an outline of the pathways of metabolism of some major steroids. A detailed account of steroid metabolism is given by Heftmann.[297] The biosynthesis of cholesterol, precursor of all steroids in animals, occurs via lanosterol as has been discussed earlier (Section 3.1.1.). This is converted to pregnenolone (5-pregnene-3β-ol-20-one) and from this precursor the pathways diverge. With respect to the qualitative and quantitative nature of urinary steroid mixtures, three strikingly different normal patterns can be distinguished: adult male, pregnant female and newborn infant.

The principal components in the adult male (and non-pregnant female) profile are reduction products of the adrenocortical hormones, e.g. tetrahydrocortisone (5β-pregnane-3α,17α,21-triol-11,20-dione), tetrahydrocortisol (5β-pregnane-3α,11β,17α,21-tetrol-20-one), cortolone (5β-pregnane-3α,17α,20α,21-tetrol-11-one) cortol (5β-pregnane-3α,11β,17α, 20α,21-pentol), and 17-ketosteroids, androsterone (5α-androstan-3α-ol-17-one) and aetiocholanolone (5β-androstan-3α-ol-17-one) with their 11-oxygenated analogues, and dehydroepiandrosterone (5-androsten-3β-ol-17-one: DHA). With the exception of DHA 3β-sulphate these are solubilized as the glucuronic acid conjugates.

The urinary steroids of pregnancy differ from those described in the elevation of several steroids - notably oestriol (1,3,5(10)-oestratriene-3,16α,17β-triol) and pregnanediol (5β-pregnane-3α,20α-diol). The involvement of the foetus in the production of estrogens has been long appreciated, and the urinary estriol level has been taken as an index of foetal viability. Other oestrogens are found as lower concentrations. Pregnanediol is the principal metabolite of progesterone, which is secreted in large amounts by the placenta. During intra-uterine life the foetus and placenta function as specialised compartments in an integrated "foeto-placental" unit. The interrelationship of these, in particular in their separate and complementary function in the biogenesis of oestrogens and progestagens, has been extensively investigated and is the subject of several reviews.[423-425] The broad outlines of this relationship are shown in Scheme 4.2. The transfer of substrates and metabolites from placenta to foetus and vice versa is primarily by umbilical vein and artery. The amniotic fluid shows steroid levels

characteristic of both placenta and foetus. The maternal circulation is interfaced to the foeto-placental unit *via* the placental barrier and during the prenatal period the interaction of foetus, placenta and mother is reflected by the steroid metabolites which appear in the mother's urine.

The pattern of urinary steroids of the newborn differs substantially from those described above: (i) the major metabolites possess a Δ^5-3β-ol structure; (ii) an oxygen function at C(16), usually a 16α-hydroxy group is a prominent feature of many metabolites; (iii) sulphate conjugation predominates; (iv) a higher proportion of the steroids in the newborn, particularly polar metabolites of cortisol, are excreted in the free form; (v) DHA, androsterone and aetiocholanolone are not detectable or occur only in trace amounts; (vi) urinary levels of THE and THF, excreted in large amounts by adults, are low in infant urine. Table 2.1. lists some of the steroids so far identified in infant urine.

The human foetal and newborn adrenal gland is much larger in relation to body weight than in the adult. This gland undergoes involution after birth, reducing the size of the large foetal zone in relation to the cortex.[44] This foetal structure is probably responsible for the origin of the large quantities of Δ^5-3β-hydroxysteroids which are subsequently modified by the infant liver. The maturation of the initially weak enzyme system responsible for the production of 4-en-3-one structures from Δ^5-3-hydroxysteroids and the decrease in the activity of the foetal zone of the adrenal account for the decrease in amount of Δ^5-3β-hydroxy-steroids and the emergence of a more "adult" distribution at about the sixth month of life. A very active 16α-hydroxylase system in the newborn accounts for the 16α-hydroxylated-Δ^5-3β-hydroxy steroids and notably for the low concentrations of 17-ketosteroids common in adult urine in favour of 16α-hydroxy analogues (*e.g.* 16α-hydroxy-DHA). The infant adrenal produces adequate supplies of corticosteroid hormones from birth.

From this brief account, steroid metabolism and the pattern of urinary steroid excretion in early infancy is highly complex and undergoes continuous change during the first six months of life. This background of constant change and the normal interindividual variations in steroid excretion greatly complicate the recognition of deviations from "normal" metabolism resulting from disease conditions or inborn errors in steroid metabolism (*e.g.* 21-hydroxylase deficiency, Δ^5-3β-dehydrogenase-isomerase deficiency, 11β-hydroxylase deficiency and 17α-hydroxylase deficiency).

Table 4.2. Some steroids present in the urine of the human newborn.

Androstane Series

diol

5-Androstene-3β,17β-diol[132,225,424,427]

5-Androstene-3β,17α-diol[225,424,427,443]

diolone

5-Androstene-3β,16α-diol-17-one[132,225,432,433,437,442-444]

5-Androstene-3β,16β-diol-17-one[132,434,443]

5-Androstene-3β,15α-diol-17-one[132,443]

5-Androstene-3β,17β-diol-16-one[432,437,441-444]

triol

5-Androstene-3β,16α,17β-triol[132,225,428-431,437,442-444]

5-Androstene-3β,16β,17β-triol[431]

triolone

5-Androstene-3β,15β,16α-triol-17-one[441]

5-Androstene-3β,16β,18-triol-17-one[441]

tetrol

5-Androstene-3β,16β,17β,18-tetrol[441]

5-Androstene-3β,15β,16α,17β-tetrol[441]

5-Androstene-3β,15ξ,16ξ,17β-tetrol[441]

Pregnane Series

5,16-Pregnadiene-3β-ol-20-one[225,426]

diol

5β-Pregnane-3α,20α-diol[132,225]

diolone

5β-Pregnane-3α,6α-diol-20-one[132,225,426]

5β-Pregnane-3α,16α-diol-20-one[132,225,426,442,443]

5-Pregnene-3β,16α-diol-20-one[225,428,435,437,444]

5-Pregnene-3β,21-diol-20-one[132,225,428,437-440,444]

triol

5-Pregnene-3β,20α,21-triol[132,426,442,443]

5β-Pregnane-3α,6α,20α-triol[132]

5β-Pregnane-3β,16α,20α-triol[132]

triolone

5-Pregnene-3β,16α,21-triol-20-one[426]

tetrol

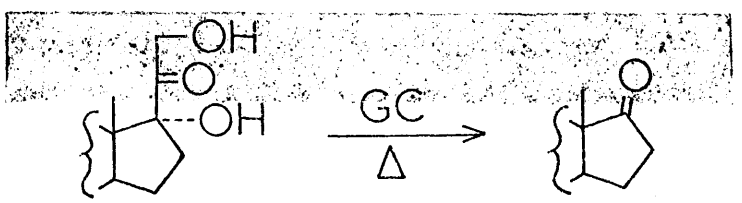
5-Pregnene-3β,16α,20α,21-tetrol[426]

5-Pregnene-3β,16ξ,17α,20ξ-tetrol[426]

4.2. Oxime Derivatives of Ketosteroids in Open Tubular Gas Chromatographic Analysis.

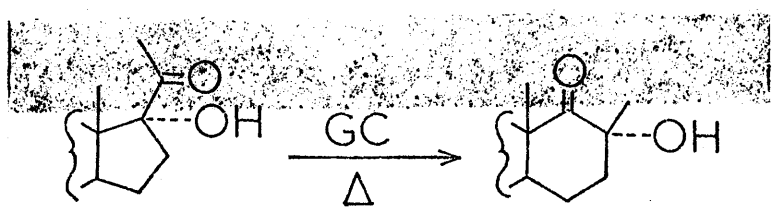
4.2.1. Derivatives and the gas phase analysis of steroids. The early 1960's saw a rapid increase in the use of g.c. in steroid analysis. This was accompanied by a demand for suitable derivatives. While many of the steroid hormones and metabolites are stable enough for direct g.c., it is generally more satisfactory to convert them to derivatives that are less polar and less liable to undergo adsorption or to be adversely affected by gas chromatographic conditions. The trimethylsilyl (TMS) ethers were first applied to steroids by Luukkainen et al.[346] These derivatives, which have excellent chromatographic properties, are now almost ubiquitous in steroid analysis. Acetates have been applied[446] but are somewhat handicapped by long retention times. Trifluoroacetates, with correspondingly shorter analysis times,[447] have proved particularly useful as selective derivatives for electron capture detectors.

There are, moreover, steroids for which the formation of derivatives is essential: the major examples are in the corticosteroid group. These compounds consist in the adrenocortical hormones, their immediate precursors and principal metabolites and constitute a critically important class. Under conditions necessary for g.c. thermal decompositions and rearrangements frequently occur. Thus the dihydroxyacetone side chain typical of cortisone is largely degraded during g.c., affording several products, notably the corresponding 17-ketone as shown below.[448]



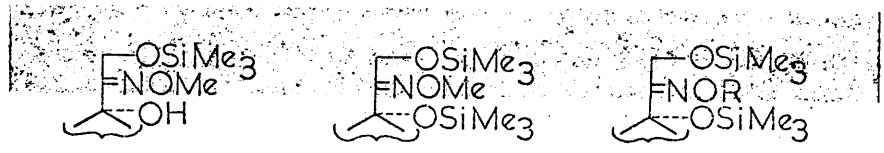
Scheme 4.3.

The related 20,17-ketols are also unstable, undergoing rearrangement to D-homosteroids such as below.

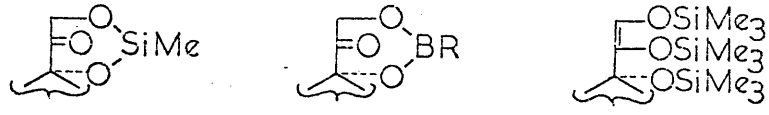


Scheme 4.4.

Protection of the dihydroxyacetone side chain is not satisfactorily achieved by trimethylsilylation of the hydroxyl groups. While chemical oxidation of corticosteroids produces products which are amenable to g.c.,[449,450] a number of effective derivatives have been developed, as exemplified by the structures below (references in brackets).



(451) (452) (442)



(453,454) (455-457) (112,458,459)

In addition to the gas chromatographic properties of derivatives, their mass spectrometric behaviour is important in g.c.-m.s. applications. Baillie et al. have evaluated the above derivatives and others for their usefulness in corticosteroid g.c.-m.s.[460] Among these, the methyloxime-TMS ethers, introduced by Gardiner and Horning in 1966,[451] and homologous alkyloxime-TMS ethers remain the most convenient for application to urinary steroid samples. Oximation is carried out first and is followed by trimethylsilylation, preferably under conditions suitable for reaction of the sterically hindered 11β- and 17α-hydroxyl groups. The prior conversion of reactive ketone groups to their oximes prevents the uncontrolled formation of enol-TMS derivatives in the subsequent silylation step.[461] The resulting mixture of TMS ethers (of non-ketonic steroids and steroids with the unreactive 11-keto group) and oxime TMS ethers is most suitably analysed by programmed temperature g.c. because of the wide range of retention index values represented. In the current brief study O-methyl, O-ethyl, O-sec-butyl and O-isopentyloxime - TMS ether derivatives of several ketosteroids were prepared and chromatographed on packed and open tubular columns. The occurrence of syn and anti isomers and the associated retention index differences (dI) were observed with particular reference to the structure of the parent steroid. These alkyloxime derivatives were examined in the light of the usefulness of these

derivatives in the elucidation of the composition of complex mixtures of ketosteroids by open tubular gas chromatography.

4.2.2. Experimental. Steroids were obtained commercially, mainly from Ikapharm (Ramat-Gan, Israel) and Sigma London Chemical Company (London, S.W.6, London). Alkyloxyamine hydrochloride reagents were obtained as follows: O-methylhydroxylamine hydrochloride and O-ethylhydroxylamine hydrochloride (Eastman Organic Chemicals, Rochester, New York, U.S.A.); O-sec-butylhydroxylamine hydrochloride and O-isopentylhydroxylamine hydrochloride (Dr. T.A. Baillie, Department of Chemistry, The University, Glasgow); O-benzylhydroxylamine hydrochloride (Aldrich Chemical Company, Inc., Milwaukee, Wis.).

Alkyloxime-TMS ether derivatives were prepared by taking 100µg of steroid in 100µl of a solution of the particular alkylhydroxylamine · HCl (40mg/ml) in freshly distilled pyridine and allowing the mixture to stand at 60°C overnight. 100µl of benzene was added to the reaction mixture and the solvent removed under a stream of dry nitrogen. 100µl of dry pyridine was added to the residue and 200 µl of a solution of 1:1 (v/v) hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) and the mixture was kept at 60°C for one hour. Solvent and excess reagent were removed under a stream of dry nitrogen. The residue was extracted with 'Nanograde' hexane and the volume of solvent adjusted to give approximately 1µg/µl for gas chromatography.

Packed column gas chromatography was performed on a Perkin-Elmer Model 881 gas chromatograph equipped with a 9ft. 1% OV-1 column and flame ionisation detector. Open tubular gas chromatography was performed on a Perkin-Elmer F11 gas chromatograph adapted as described in Section 2.2.7. Retention indices were calculated graphically by semi-logarithmic interpolation from the retention times of co-injected n-alkanes, n-$C_{24}H_{50}$ and n-$C_{32}H_{66}$.

G.c.-m.s. analysis was carried out on a 40m Silanox-type glass (PLOT) OV-1 column installed with make-up gas adaptor in the LKB 9000 as described in Section 2.3.3. with separator temperature and ion source temperature 270°C, electron energy 70eV.

4.2.3. Results and discussion. As was mentioned, the derivatives of choice for steroidal ketones of various structures have been the substituted oximes. Polar simple (unsubstituted) oximes, may be connected to non-polar O-trimethylsilyloximes[462-464] or O-acetyloximes.[464] The O-methyloxime (MO), introduced by Fales and Luukkainen[110] is recommended by their stability, ease of formation and good g.c. properties. The mixed MO-TMS derivatives of Thenot and Horning have

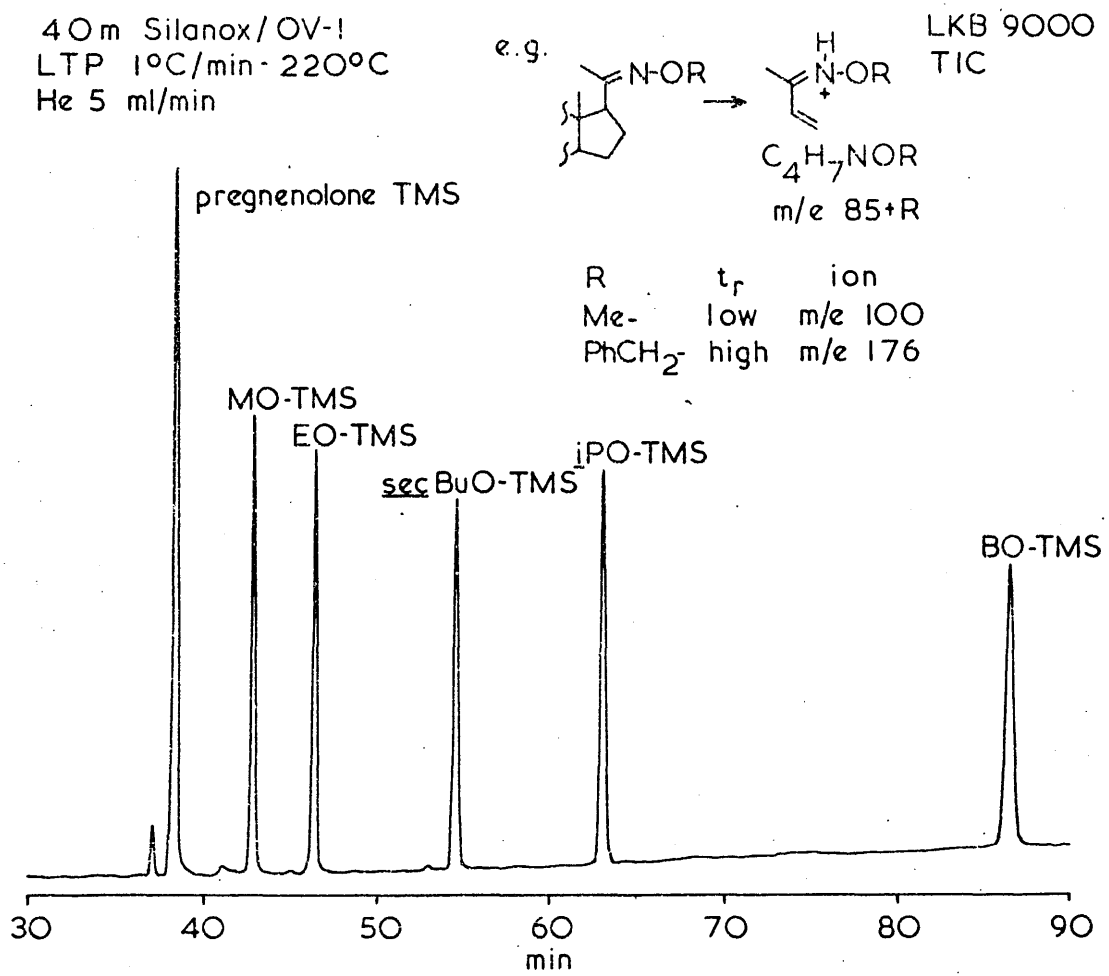


Figure 4.2. Chromatogram of a mixture of the trimethylsilyl (TMS) ether and alkyloxime-trimethylsilyl ether derivatives of pregnenolone demonstrating the retention index increments for the series of alkyl substituents. Chromatographic conditions: 40m x 0.5mm Silanox-type (PLOT) OV-1 column, installed in the LKB 9000, linear temperature programme 1°/min from initial temperature 220°C, helium carrier gas 5ml/min. Insert scheme shows prominent feature of 20-alkyloxime electron impact (EI) mass spectra: source temperature 270°C, electron energy 70eV.

been applied to mixtures of hydroxy- and ketosteroids such as are found in the urinary steroid samples.[465]

As was discussed in Section 1.4.5., implicit in the doctrine for derivatives for g.c.-m.s. is not only the straightforward improvement of g.c. behaviour but the production of structurally relevant alterations in the g.c. and m.s. characteristics. The application of alkyloximes in ketosteroid analysis aptly demonstrates this. Whereas the retention index increment associated with the formation of the MO is small the analogous O-benzyloxime (BO) affords complete separations of ketosteroids from related hydroxysteroids and steroids possessing unreactive keto groups.[444,465] The large retention increments associated with BO formation are disadvantageous because of the conditions required for the analysis of diketonic steroids. This is particularly important in multi-component mixtures. To avoid this difficulty a number of alkyl substituents producing intermediate retention times have been employed.[111,442] These derivatives also permit the recording of a range of profile separations from complex mixtures of hydroxy- and ketosteroids which strengthen interpretations based on g.c.-m.s. (see for example Figure 4.4.).

Figure 4.2. illustrates the retention increments associated with a series of alkyloxime derivatives (from methyl to benzyl) and also the alteration of a fragmentation of these derivatives associated with the 20-keto group in 5-pregnen-3β-ol-20-one (pregnenolone). The 3β-hydroxy group of pregnenolone has been derivatised as the TMS ether, and the retention index increments (dI values) for the oximation of the 20-keto function [O-methyl (+78), O-ethyl (+157), O-sec-butyl (+272), O-isopentyl (+313) and O-benzyl (+788)] are illustrated. The ring D fragment together with the molecular ion shows a corresponding mass increment as R is altered from methyl to benzyl. The prominent features of the mass spectra of these derivatives are summarized in Table 4.2. The spectra of these compounds show several features common to O-alkyloximes of other ketosteroids: prominent molecular ions and characteristic loss of the N-alkoxy radical.[442] In addition the ring D cleavage provides a feature characteristic of the O-alkyloxime of 20-ketosteroids,[467,468] an ion comprising the sidechain together with the oxime grouping, C(16) and C(17) with a hydrogen transfer from the nucleus.

Table 4.3. shows retention data for several representative steroids possessing the 3-one,4-en-3-one,16-one -unsubstituted at C(17), 17-one, 20-one and 3,20-dione structures as alkyloxime-TMS ether derivatives ranging from MO-TMS through BO-TMS. Isomers of the syn/anti type have been observed for some ketosteroid MO derivatives[451,467,469-473] and for higher alkyloxime derivatives.[111,442,474] This isomerism is confirmed here,

Table 4.2. Retention indices* and salient features of the mass spectra** of trimethylsilyl (TMS) ether and alkyloxime-trimethylsilyl ether derivatives of pregnenolone. See Figure 4.2.

Derivative	I*	I	M^+	Base	$(M-15)^+$	$(M-90)^+$	129^+	$(M-129)^+$	$(M-OR)^+$	$C_3H_6NOR^+$	$C_4H_7NOR^+$	Other ions
TMS ether	2738		388	129	373	298	129	259	-	-	-	332 283 241 145 121 75 73 (18)(20)(15)(15)(18)(33) (65)
Methyloxime-TMS ether	2816	78	417 (20)	100	402 (46)	327 (11)	129 (61)	288 (40)	386 (55)	87 (67)	100 (100)	312 296 239 73 (31)(22)(25) (86)
Ethyloxime	2875	137	431 (21)	114	416 (40)	341 (7)	129 (58)	302 (17)	386 (55)	101 (91)	114 (100)	296 239 73*** (18)(22)(110)
sec-Butyloxime-TMS ether	3010	272	459 (17)	129	444 (24)	369 (3)	129 (100)	330 (11)	386 (40)	129 (100)	142 (51)	296 239 73 (12)(12)(100)
Isopentyloxime-TMS ether	3051	313	473	156	468 (55)	383 (5)	129 (86)	344 (22)	386 (58)	143 (30)	156 (100)	296 239 73*** (21)(28)(180)
Benzyloxime-TMS ether	3526	788	493 (12)	163	488 (21)	403 (11)	129 (42)	364 (10)	386 (71)	163 (100)	176 (48)	239 91*** 73*** (11)(350) (110)

* Gas chromatographic conditions as in Figure 4.2.
** Mass spectrometer conditions: LKB 9000, source temperature 270°C, electron energy 70eV.
*** Base peak most abundant above m/e 100.

Table 4.3. Retention index values for some steroid alkyloxime-trimethylsilyl ether derivatives on packed* and open tubular** columns.

Steroid	Derivatives: Packed column* retention indices (open tubular column** retention indices in brackets) TMS	MO-TMS	EO-TMS	sec-BuO-TMS	i-PO-TMS
5α-Androstane-3α-ol-17-one (androsterone)	2451 (2470)	2519 (2534)	2575 (2589)	2693 (2698)	2828 (2836)
5-Pregnene-3β-ol-20-one (pregnenolone)	2688 (2710)	2770 (2792)	2830 (2852)	2953 (2983)	3082 (3110)
5β-Androstane-17β-ol-3-one	2515 (2559)	2583 (2619/2623)	2637 (2673/2677)	2749 (2777/2785)	2883 (2909/2920)
4-Androstene-17β-ol-3-one (testosterone)	2614 (2661)	2659 (2694/2696)	2714 (2751)	2824/2840 (2856/2876)	2961/2979 (2980/3018)
5-Androstene-3β-ol-16-one	2528 (2552)	2614 (2640/2648)	2679 (2698/2706)	2806 (2822/2832)	2941/2955 (2952/2968)
5α-Pregnane-3,20-dione***	2693 (2710)	2820 (2845/2857)	2928/2937 (2952/2970)	3172/3193 (3194/3220)	3438/3464 (3461/3486)
4-Pregnene-3,20-dione (progesterone)	2759 (2785)	2854 (2887)	2970 (2993/3000)	3203/3212 (3222/3244)	3492/3499 (3492/3518)

* Packed column conditions: 9ft. 1% OV-1 coated on 100-120 mesh Gas Chrom Q installed in Perkin-Elmer Model 881 gas chromatograph equipped with flame ionisation detector, 205°C, nitrogen carrier gas 40ml/min.

** Open tubular column conditions: 50m x 0.5mm Silanox-type (PLOT) glass OV-1 column installed in Perkin-Elmer Model F11 gas chromatograph equipped with flame ionisation detector, 230°C, helium carrier gas 4ml/min.

*** Temperatures: packed column, 230°C; open tubular column, 240°C.

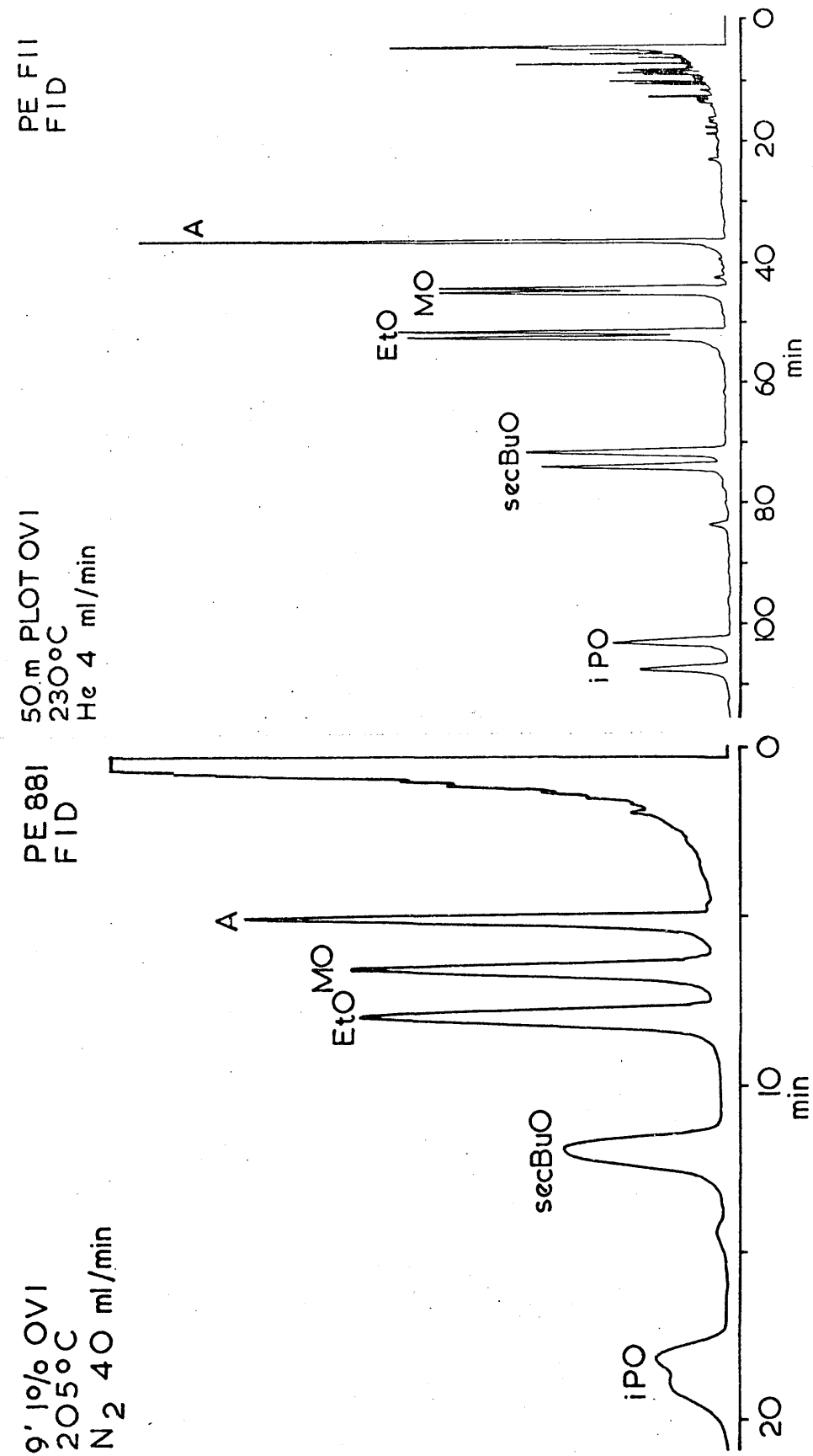


Figure 4.3. Packed (A) and open tubular (B) column separations of a mixture of 5-androsten-3β-ol-16-one as the TMS ether derivative (A) and as the O-methyl (MO), O-ethyl (EtO), O-sec-butyl (secBuO) and O-isopentyl (iPO)-oxime-TMS ether derivatives. Chromatographic conditions: A. 9ft. 1% OV-1 installed in a Perkin-Elmer Model 881 (FID), 205°C, nitrogen 40ml/min; B. 50m Silanox-type glass (PLOT) OV-1 installed in a Perkin-Elmer Model F11 (FID), 230°C helium 4ml/min.

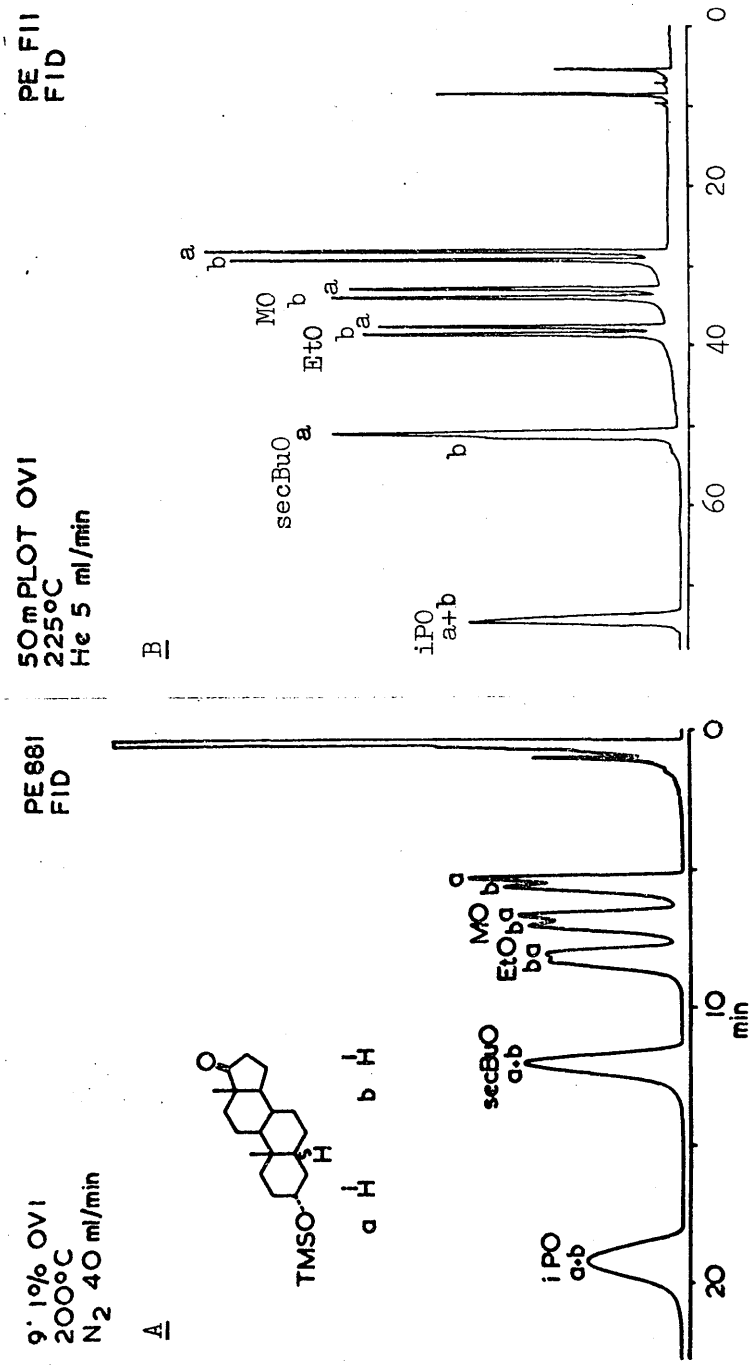


Figure 4.4. Packed (A) and open tubular (B) column separations of a mixture of androsterone (a) and aetiocholanolone (b) as the TMS ether and as the O-methyl (MO), O-ethyl (EtO), O-sec-butyl (secBuO) and O-isopentyl (iPO)-oxime-TMS ether derivatives. Chromatographic conditions : A. 9ft 1% OV-1 installed in a Perkin-Elmer Model 881 (FID), 200°C, nitrogen 40ml/min, B. 50m Silanox-type glass (PLOT) OV-1 installed in a Perkin-Elmer Model F11 (FID), 225°C, helium 5ml/min.

the retention difference associated with this being most marked in the 17-unsubstituted 16-one and where syn/anti isomerism is possible showing consistent increases in retention index difference with increasing bulk of the O-alkyl substituent. The chromatographic consequences are demonstrated by the packed and open tubular column chromatograms shown in Figure 4.3. An anomaly was observed with the occurrence of only a single peak for testosterone O-ethyloxime TMS ether contrasting with the twin peaks for MO-TMS and sec-BuO-TMS. The occurrence of several different combinations of isomeric configurations in diketone systems has been observed by t.l.c.[467] In the g.c. analysis several peaks observed in varying minor proportions to the two major peaks whose retention indices are recorded. The reaction conditions and the specific ketone structures appear to influence the proportions observed.

The occurrence of syn and anti isomers of alkyloxime derivatives is of importance in multicomponent analysis of ketosteroids particularly on columns of higher efficiency. The effect of the application of higher efficiencies with higher alkyloximes for mixtures of unknown ketosteroids may in the most disadvantageous case simply be a doubling of the number of peaks to identify. In the cases of ketone groups located at C(17) and C(20), as in most urinary steroid metabolites, a single g.c. peak is obtained,[467,473,474] even at the efficiency provided by open tubular columns. However, Figure 4.4. illustrates an unexpected effect for alkyloxime-TMS ether derivatives in the separation of the 17-ketosteroids androsterone (5β-androstane-3α-ol-17-one) and aetiocholanolone (5α-androstane-3α-ol-17-one). As the bulk of the alkyl substituent is increased the separation is reduced on both packed and open tubular columns. The separation is completely eliminated in the case of the isopentyloxime-TMS ether on a 50m open tubular column.

From these indications it would seem that the methyloxime derivatives are the best choice for simplicity in analysis by high efficiency columns of complex mixtures such as urinary steroid profiles. However, as has been mentioned, different alkyloximes may be usefully employed to provide a range of profile separations to strengthen interpretations based on g.c. and g.c.-m.s. This is illustrated by the profile separations of the urinary steroids of a normal adult male as both the methyloxime and isopentyl oxime TMS ethers shown in Figure 4.5. The identities of the large number of peaks shown in these profiles will not be discussed in detail. The distinction between ketonic and non-ketonic compounds is immediately possible and several overlapping peaks are

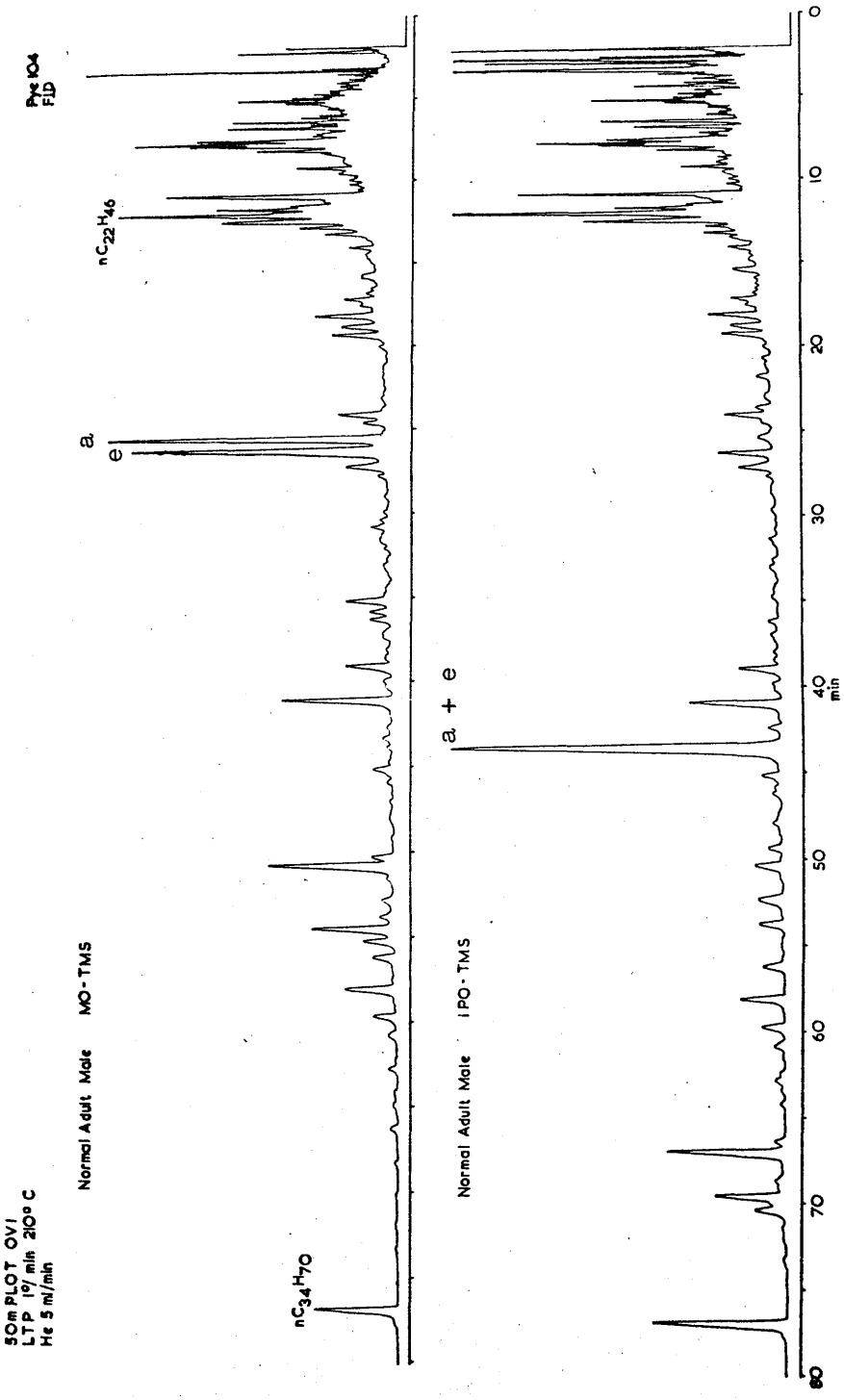


Figure 4.5. Comparison of the chormatograms of the urinary steroids of a normal adult male as the methyloxime-trimethylsilyl ether (upper) and isopentyloxime-trimethylsilyl ether (lower) on a 50m Silanox type (PLOT) glass OV-1 column installed in a Pye 104. Helium carrier gas 5ml/min, linear temperature program 1°/min from 210°C. Androsterone (a) and aetiocholanolone (e) derivatives are indicated and co-injected n-alkane standards (n-$C_{22}H_{46}$ and n-$C_{34}H_{70}$) are marked.

usefully shifted. The coalescence of the isopentyloxime - TMS ether peaks for androsterone and aetiocholanalone, the major 17-ketosteroid metabolites in the human male, is seen.

4.3. Open Tubular G.C. and G.C.-M.S. of Newborn Urinary Steroids.

4.3.1. Introduction. A large number of techniques have been developed for the analysis of steroid hormones. These vary greatly in complexity, specificity and precision. Chromatographic techniques are among the most useful and g.c. has found wide application. G.c. may be used for the estimation of one or many components in a single separation. In the former case prior fractionation of complex mixtures may be necessary to improve specificity but this may adversely affect sensitivity. The possibility of carrying out multi-component analyses has led to the concept of "metabolic profile" analysis in the study of human metabolites of various classes of compound isolated from tissues and biological fluids. Notable among such analyses have been the study of the total urinary steroids of the human newborn by Horning and co-workers.[430,437,464,475,478]

The urinary steroid profile is generally characterised by (a) isolation of a suitable analytical sample from urine; (b) conversion of the components of the sample into derivatives suitable for g.c. separation; (c) analysis (generally under temperature programming) with columns of the highest practicable efficiency; (d) conversion of data obtained on the separated components into useful qualitative and quantitative information. In this latter operation g.c.-m.s. and the application of integrated computer techniques provide the best practicable combination of sensitivity of detection and selectivity of qualitative characterisation. In the case of urinary steroids an enzymic hydrolysis (liberating steroids from conjugating acids) is followed by isolation by solvent extraction. Further separation methods may be employed but the object at this point is to obtain all steroids in the same relative concentrations as they appear in the original urine with no substances which may interfere in later stages. Subsequently "group" separations may be employed to subfractionate this steroid mixture, so as to simplfy later multicomponent analysis, and to provide additional structural information. Liquid chromatography on Sephadex gels and related materials have proved useful in this respect.[132,443] The concepts of derivative formation discussed earlier (Section 4.2.1.) are applied to their full advantage in the analysis of urinary steroids. The use of the O-alkyl-oxime TMS ether derivatives facilitates the gas phase "group separation"

of hydroxy and ketosteroids.

The efficacy of glass open tubular columns for the analysis of complex mixtures of urinary steroids was first demonstrated by Völlmin[210] but the full exploitation of this capacity awaited the development of reproducible thermostable columns. As was mentioned earlier, several laboratories closely involved in the problem of steroid analysis carried out pioneering work on the preparation of glass open tubular columns. These methods, which have been discussed earlier, have found advantageous application. The following is a brief account of the use of Silanox-type glass open tubular gas chromatographic columns in the g.c.-m.s. analysis of urinary steroids.

4.3.2. Experimental. Reference steroids were obtained from the commercial sources mentioned earlier (Section 4.2.2.). Steroid extracts from newborn urine were obtained from the laboratory of Professor E.M. Chambaz, Laboratoire d'Hormonologie, C.H.R. de Grenoble, 38400-La Tronche, France. Extractions of the urine of newborn infants was performed by Dr. R.A. Anderson by methods described elsewhere.[132] Derivatives were prepared of synthetic and natural mixtures of hydroxy- and ketosteroids using 0.4-1mg (ca.1-2.5μmole) of steroid. Mixed alkyloxime-TMS ether derivatives were prepared by a "two-step" procedure under the conditions described for MO-TMS derivatives by Thenot and Horning.[465] The mixture (approximately 500 μg) was placed in a screw-cap "Reacti-Vial" (Pierce and Warriner (U.K.) Ltd., Chester, Cheshire, CH1 4EF), and the appropriate amount of the desired alkylhydroxylamine hydrochloride (8-10mg) added. The mixture was taken up in dry pyridine (200μl) and heated for two hours at 60°C or allowed to remain at room temperature overnight. The solvent was removed under a stream of dry nitrogen and the residue treated with 100ml of trimethylsilylimidazole (TSIM: Pierce and Warriner (U.K.) Ltd.) which had been previously purified by vacuum distillation. After two hours at 100°C, 0.5ml of hexane/pyridine/hexamethyldisilazane (98:1:1 by volume) was added and the reagents were removed from the derivatized steroids by filtration through a Lipidex 5000 (Packard-Becker Co., Groningen, The Netherlands) column according to the procedure of Axelson and Sjövall.[479]

This column was prepared by gravity flow with pre-swollen gel in hexane/pyridine/hexamethyldisilazane (98:1:1) to give the dimensions 70 x 4mm (corresponding to 0.25g of dry gel), and was washed with 10ml of the solvent mixture prior to application of the sample. The sample was eluted through the column at a flow rate of 6ml/hr. A total of 2ml of effluent was collected in a small conical screw-cap vial, solvents were removed

under a stream of dry nitrogen, and the residue of reagents-free derivative was dissolved in an appropriate volume of hexane for immediate analysis by g.c. or g.c.-m.s.

Linear temperature programming (LTP) was found to be the most suitable mode for chromatographic analysis and the methylene unit[480] (MU) was employed. When a series of n-alkanes is separated in LTP mode an approximately linear relationship is observed between retention times of successive n-alkane peaks. Using these as reference substances, the MU value of an unknown can be obtained by linear interpolation between two bracketing n-alkanes according to the formula:

$$MU = n \cdot \frac{tr_x - tr_z}{tr_{z+n} - tr_z} + Z \qquad \text{(eq. 4.1.)}$$

In this expression, tr_x is the corrected retention time of the unknown and z and z+n are the carbon numbers of the bracketing n-alkanes. Adjacent even numbered alkanes were employed where possible (n = 2).

The precision of these data was of interest in view of earlier evidence (Perkin-Elmer F11 and LKB 9000 systems: Section 3). The precision of retention data of the Pye 104 system was evaluated in isothermal and temperature programme modes and the results are shown in Table 4.4. These results indicate that the performance of this chromatographic system is somewhat better and that the data from both LTP and isothermal modes are of comparable precision. At low programming rates ($\lesssim 1^{\circ}$/min) the linear relation between retention time and carbon number of n-alkane standards is only approximate. This quasi-linear behaviour results in poor accuracy where bracketing n-alkanes are widely separated (see MU for n-$C_{26}H_{54}$ in Table 4.4.). Adjacent n-alkanes are thus the most suitable standards and a mixture of even numbered n-alkanes (C_{18} to C_{36}) was normally employed as a co-injected standard. Where the use of this was impractical (e.g. for minor components of profile mixtures) relay calculations were performed using MU values of major components obtained in the normal way or by means of more widely spaced n-alkanes, the intermediate reference t_r values being calculated from the ratios of standard n-alkane t_r values obtained under identical conditions.

4.3.3. Application to synthetic and natural steroid mixtures. The accuracy of MU data obtained on open tubular columns in this laboratory was compared with literature data. A mixture of hydroxy- and ketosteroids was derivatised in the form of methyloxime-TMS ethers (MO-TMS) and isopentyloxime-TMS (iPO-TMS) ethers and chromatographed on both packed and

Table 4.4. Retention data for androsterone and aetiocholanolone as TMS ethers and n-$C_{26}H_{54}$ on a Silanox-type (PLOT) glass OV-1 column under isothermal and temperature programme conditions.

	Androsterone	Aetiocholanolone	n-$C_{26}H_{54}$
a) Isothermal 230°C (Kováts retention indices (I)*)			
	2479.9	2495.7	2601.3
	2481.1	2497.4	2600.6
	2480.2	2496.1	2601.6
	2479.1	2495.9	2600.7
	2479.7	2495.6	2601.9
$\bar{x}$	2779.9	2496.3	2601.3
6	0.84	0.76	0.49
%6	0.033	0.030	0.018
b) Linear temperature programme 220°C/1°/min (Methylene units (MU)**)			
	24.698	24.847	25.838
	24.701	24.855	25.841
	24.715	24.869	25.839
	24.709	24.858	25.844
	24.708	24.861	25.850
$\bar{x}$	24.706	24.858	25.842
6	0.006	0.008	0.005
%6	0.027	0.032	0.018
c) Linear temperature programme 220°C/1°/min (Methylene units (MU)**)			
	24.861	25.015	25.926
	24.869	25.019	25.930
	24.863	25.015	25.928
	24.867	25.019	25.931
	24.879	25.024	25.931
$\bar{x}$	24.868	25.018	25.930
6	0.007	0.004	0.002
%6	0.028	0.014	0.008

* Calculated by semilogarithmic interpolation (algebraically from bracketing n-alkanes, n-$C_{24}H_{50}$ and n-$C_{28}H_{58}$).

** Calculated by linear interpolation (algebraically from bracketing n-alkanes, n-$C_{24}H_{50}$ and n-$C_{28}H_{58}$).

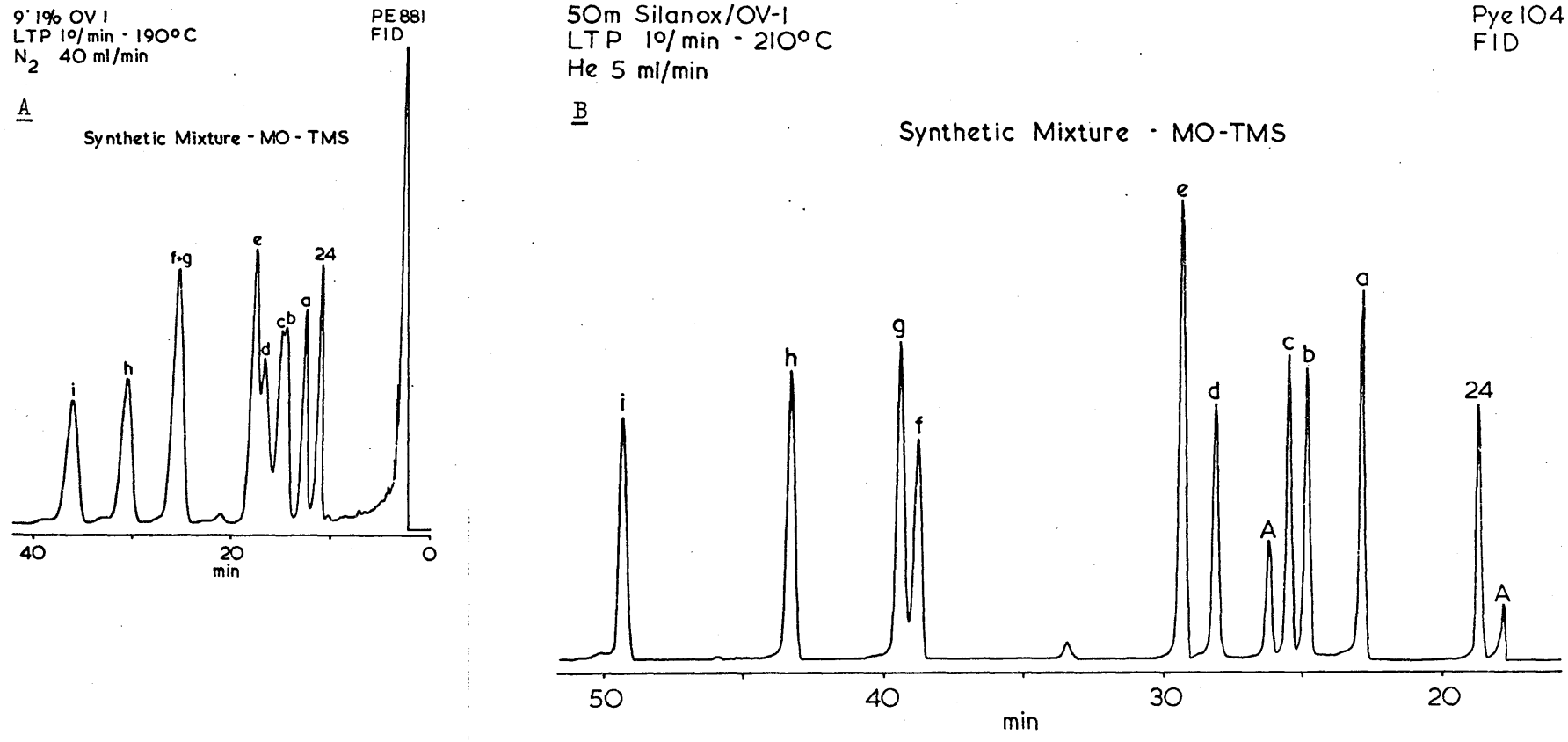


Figure 4.6. Chromatogram of the separation of a synthetic mixture of hydroxy and keto steroid standards derivatised as the trimethylsilyl (TMS) ethers, methyloximes, and methyloxime trimethylsilyl ethers (MO-TMS) on a packed (A) and a Silanox-type glass column (B). n-Tetracosane (24) is included as internal standard and two prominent peaks (A) result from artifacts entering in the derivatisation process. (a) 5α-pregnan-20-one, (b) androsterone, (c) aetiocholanolone, (d) dehydroepiandrosterone, (e) 5α-androstane-3β,17β-diol, (f) pregnenolone, (g) 5α-pregnan-3β-ol-20-one, (h) THS (tetrahydrocortexolone), (i) THE (tetrahydrocortisone).

open tubular columns. The MU values for these (unresolved components being assigned a single value) are recorded in Table 4.5. with data from other sources for comparison. Two sets of standard data of remarkable mutual consistency are shown. Luyten[225] and German and Horning,[128] both employing glass open tubular columns with methylsiloxane phases, show standard data which rarely differ by more than 0.02MU. The correspondence in the present experimental work is less close, with consistent differences of 0.1-0.2MU. There are, however, satisfactory consistencies within the series.

Delaforge et al.[481] have demonstrated and discussed the strong dependence of the absolute value and reliability of MU data on the initial temperature in the programme. Their evidence suggests that best accuracy pertains at lower initial temperatures. However, initial temperature is also governed by acceptable analysis time and sensitivity of detection for a particular sample. A relatively high initial temperature, 220°C, was selected for the urinary steroid mixtures, particularly for the iPO-TMS derivatives.

The utility of glass open tubular columns in g.c.-m.s. analysis of hydroxy- and ketosteroids is illustrated by the chromatogram of TMS and methyloxime TMS derivatives of reference steroids shown in Figure 4.6. (packed column chromatogram A). The components for which methyloxime formation is essential are the dihydroxyacetone-type steroids (peaks h and i).

Characterisation of the steroids depends on the accurate determination of retention parameters - and on the correlation of the g.c. data with mass spectra recorded at defined retention times. The establishment of relationships between reference steroids is pre-requisite data for the identification of steroids in biological samples. Salient features of data for the compounds represented in Figure 4.6. are summarized in Table 4.6. Some salient diagnostic points are summarized below.

(i) The methyloximes (of monoketones) are distinguished by their odd molecular weights: where the molecular ion is weak the ions of m/e $(M-31)^+$, due to loss of methoxy radical, and of m/e $(M-121)^+$ due to further loss of trimethylsilanol, are directly helpful.

(ii) The isomeric steroids androsterone (b) and aetiocholanolone (c) give rise to similar mass spectra, and their gas chromatographic properties provide the essential distinction.

(iii) The characteristic ions produced by 20-ketosteroid methoximes at m/e 100 and 87 (cf. Figure 4.2.) are prominent for 5α-pregnan-20-one (a) and pregnenolone (b) derivatives: in the latter case the ions at m/e 129 and 288, $(M-129)^+$ are also abundant, indicating the Δ^5-3-OTMS grouping.

Table 4.5. Methylene unit (MU) values for some standard urinary steroids as methyloxime-TMS ethers and isopentyloxime-TMS ethers on packed and open tubular columns.

Compound	MO-TMS				i PO-TMS		
Column:	1% OV-1	Silanox OV-1	WCOT OV-1[a]	Silanox OV-1[b]	1% OV-1	Silanox OV-1	I_{270} 1% OV-1[c]
Pregnanediol (5β-pregnane-3α,20α-diol)	27.52	27.79	27.85	27.86	27.51	27.80	2796
Pregnanetriol (5β-pregnane-3α,17α,20α-triol)	27.92	28.11	28.20	28.19	27.91	28.13	2824
Androsterone (5α-androstane-3α-ol-17-one)	25.01	25.26	25.32	25.30	28.31	28.58	2861
Etiocholanolone (5β-androstane-3α-ol-17-one)	25.19	25.42	25.44	25.45			2863
Dehydroepiandrosterone (5-androstene-3β-ol-17-one)	25.70	25.90	25.96	25.99	28.94	29.33	—
11-Hydroxyetiocholanolone (5β-androstane-3α,11β-diol-17-one)	27.06	27.19	27.34	27.32	29.85	30.04	3000
Cortolone (5β-pregnane-3α,17α,20α,21-tetrol-11-one)	30.36	30.59	30.67	30.65	30.43	30.58	3083
α-Cortol (5β-pregnane-3α,11β,17α,20α,21-pentol)	31.11	31.33	31.45	31.42	31.17	31.32	3144
THE (5β-pregnane-3α,17α,21-triol-11,20-dione)	29.64	29.86	29.77	29.77	32.19	32.31	3234
THA (5β-pregnane-3α,21-diol-11,20-dione)		29.79	29.89	29.88	32.58	32.69	3283
THF (5β-pregnane-3α,11β,17α,21-tetrol-20-one)	30.19	30.30	30.39	30.38			3270
aTHF (5α-pregnane-3α,11β,17α,21-tetrol-20-one)		30.41	30.49	30.49	32.79	32.83	3292
Conditions:	190°C 1°/min	220°C 1°/min	220°C 1°/min	200°C 1°/min	190°C 1°/min	220°C 1°/min	isothermal 270°C

Literature reference data:

a) J.A. Luyten, Gas-liquid chromatography of steroids with glass capillary columns: a breakthrough, Drukkerij, J.H. Pasmans, 'S-Gravenhage, 1973.

b) A.L. German and E.C. Horning, J. Chromatogr. Sci., 11 (1973) 76.

c) T.A. Baillie, Ph.D. Thesis, University of Glasgow, 1973.

Table 4.6. Retention indices and salient mass spectral features of the methyl oxime-trimethylsilyl ether (MO-TMS) derivatives of the standard compounds separated in the chromatogram shown in Figure 4.6.

Figure reference	Compound	MU*	$M^{\cdot+}$ (%)	Base peak	Prominent fragment ions**						
a	5α-Pregnan-20-one	24.90	331 (1)	100	300 (33)	243 (6)	232 (4)	217 (6)	173 (5)	119 (5)	87 (52)
b	Androsterone (5α-androstane-3α-ol-17-one)	25.35	391 (1)	270	360 (50)	300 (7)	213 (12)	107 (18)	75 (55)		
c	Aetiocholanolone (5β-androstane-3α-ol-17-one)	25.50	391 (1)	270	360 (45)	300 (4)	213 (13)	107 (16)	75 (47)		
d	Dehydroepiandrosterone (5-androstene -3β-ol-17-one)	26.05	389 (10)	129	358 (38)	299 (13)	284 (16)	268 (45)	260 (39)	105 (12)	73 (31)
e	5α-Androstane-3β,17β-diol	26.30	436 (11)	129	346 (18)	331 (11)	256 (16)	241 (26)	215 (22)	73 (73)	75 (73)
f	Pregnenolone (5-pregnene -3β-ol-20-one)	27.95	417 (20)	100	402 (46)	386 (55)	312 (31)	288 (40)	239 (25)	129 (61)	87 (67)
g	5α-Pregnan-3β-ol-20-one	28.10	419 (10)	388	404 (15)	298 (8)	243 (11)	107 (11)	100 (71)	87 (35)	75 (37)
h	THS (5β-pregnane-3α,17α,21-triol-20-one)	28.75	595 (23)	564	474 (22)	384 (15)	255 (23)	244 (17)	147 (38)	103 (38)	73 (375)***
i	THE (5β-pregnane-3α,17α,21-triol-11,2-dione)	29.75	609 (65)	488	578 (30)	506 (40)	398 (38)	147 (35)	103 (34)	73 (360)***	

* Chromatographic conditions as noted in Figure 4.6.
** Mass spectrometer conditions: LKB 9000, source temperature 270°C, electron energy 70eV.
*** Abundance determined for base peak m/e 100 or above.

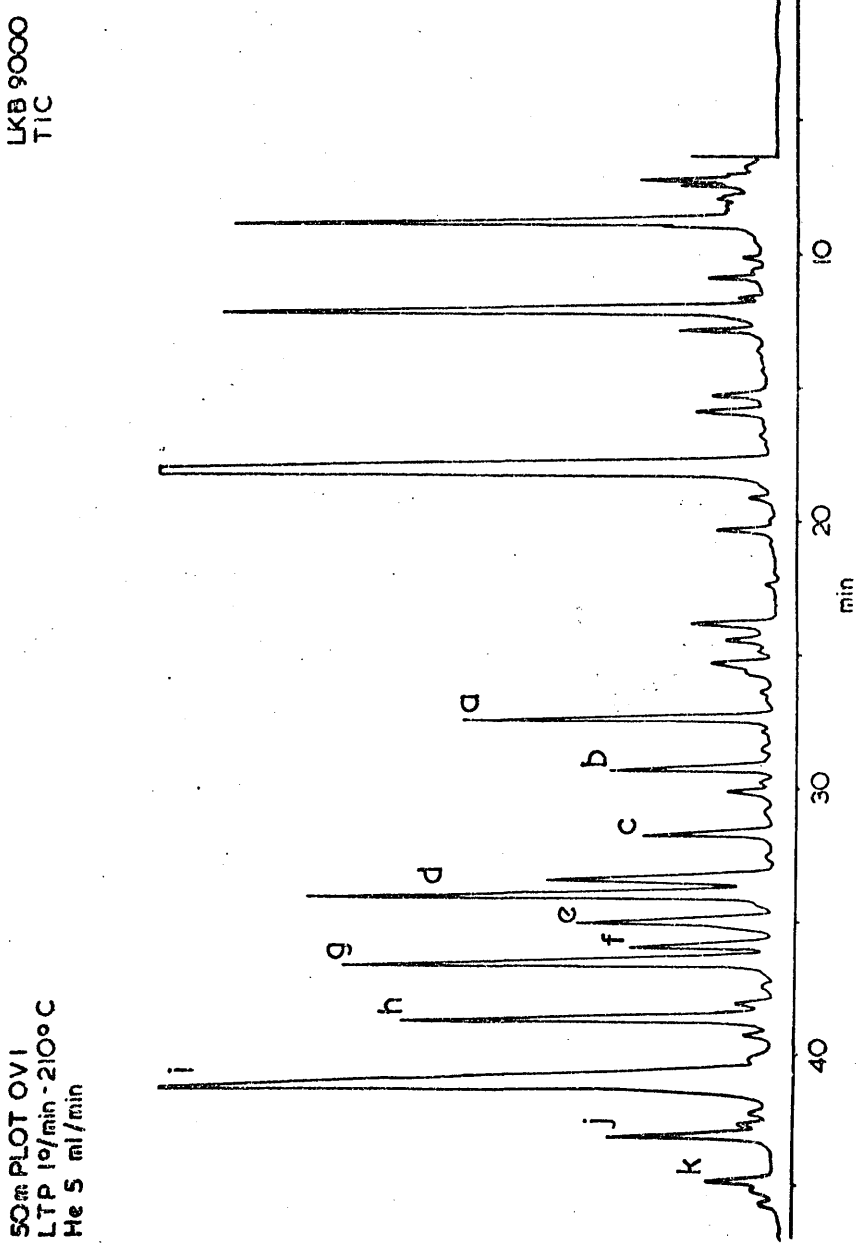


Figure 4.7. TIC chromatogram of the separation of pooled human newborn urinary steroids as the isopentyloxime-TMS ether derivatives. G.c. conditions: 50m Silanox-type glass (PLOT) OV-1 column, linear temperature programme 1°/min from 210°C, helium carrier gas 5ml/min. M.s. conditions: LKB 9000, separator and ion source 270°C, electron energy 70eV.

In the spectrum of 5α-pregnan-3β-ol-20-one methoxime TMS ether, the ion $(M-31)^+$ is predominant, but that of m/e 100 is also of high abundance.

(iv) The base peak of m/e 129 in (e) does not arise from a Δ^5-3-OTMS group, but largely from the 17β-OTMS group. The molecular ion indicates a saturated steroid, and the absence of a peak of m/e $(M-129)^+$ is in accord with this.

(v) Mass spectra of the corticosteroid derivatives (h and i) show prominent molecular ions and a preponderance of ions of m/e $(M-31)^+$, $(M-121)^+$ and $(M-211)^+$ resulting from losses of methoxyl and trimethylsilanol moieties. Ions of m/e 103 and - in the case of THE (i) - $(M-103)^+$ arise from the primary OTMS group at C(21).

A few general observations arise from the consideration of this trial. It may be said that the identification of the molecular ion is of initial importance. Recognition of the probable origin of the principal fragment ions must then be based on careful judgement of the spectrum as a whole, assessment of the compatibility of the retention data, and comparison with data for reference compounds of as similar structure as possible.

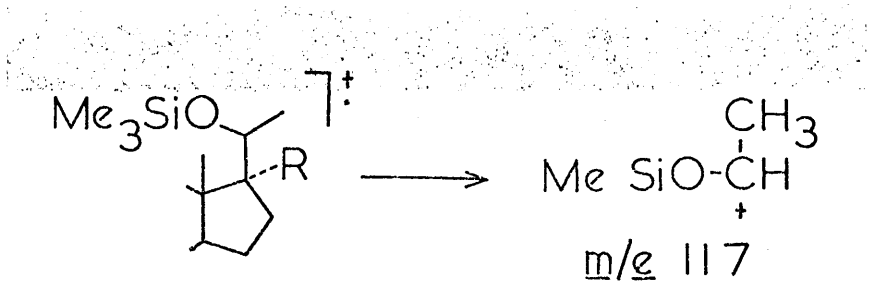
The facility demonstrated above was applied to the analysis of a natural mixture of urinary steroids. The pooled urinary steroids of normal human newborns were derivatised as the isopentyloxime-TMS ether derivatives and these were examined by open tubular g.c.-m.s. The TIC chromatogram obtained is illustrated in Figure 4.7. The relevant m.s. data for the annotated peaks in Figure 4.7. are cited in Table 4.7.

The materials eluted within the first 20minutes are endogenous, non-steroidal materials and contaminants arising in sample preparation and derivatisation. Notable is the large peak eluting at about 18 minutes (MU = 2514) which is a contaminant from the silylating reagent. The next portion of the chromatogram, up to the point of elution of the first prominent ketosteroid (16α-hydroxy-DHA) is the region containing the bulk of hydroxysteroids. The first prominent hydroxy steroid (a) is a 5-androstenetriol, showing a base peak at m/e 129, characteristic of of Δ^5-3-OTMS structure (Scheme 2.4.) and prominent ions corresponding to successive losses of trimethylsilanol: m/e 432, $(M-90)^+$; m/e 342, $(M-2 \times 90)^+$; m/e 252, $(M-3 \times 90)^+$. A second androstenetriol, 5-androstene-3β,16α,17β-triol (b) shows g.c. and m.s. characteristics very similar to this compound in other newborn steroid profiles.[132,450,473] A tentative assignment of a pregnanetriol structure for (c) which unexpectedly lacks molecular ion confirmation is based on the base peak of m/e 117 characteristic of 20-trimethylsilyloxy structures.

Table 4.7. Methylene units (MU) and salient mass spectral features of selected constituents of pooled human newborn urinary steroids as their isopentyloxime-trimethylsilyl ether (iPO-TMS) derivatives.

Figure reference*	MU*	M^{+}	Base peak	Prominent fragment ions* (%)					Proposed structures
a	27.85	522	129	432 (38)	342 (32)	252 (49)	213 (52)		5-androstenetriol
b	28.40	522	129	437 (22)	119 (26)	169 (14)	147 (38)		5-androstene-3β,16α,17β-triol
c	29.20	-	117	495 (9)	431 (80)	251 (24)	191 (40)	147 (68)	possible pregnanetriol
d	29.76 **	533	129	446 (60)	356 (34)	266 (90)	145 (40)		5-androstene-3β,16α-diol-17-one (16α-hydroxy-DHA)
	29.93 **	533	129	496 (38)	356 (29)	266 (68)	145 (32)		
e	30.27	621	147	429 (19)	355 (34)	266 (19)	157 (14)	129 (31)	5-androstene-3β,11β,16-triol-17-one
f	30.55	458	129						cholesterol
g	30.76	533	129	446 (57)	356 (34)	266 (40)	230 (52)	160 (64)	5-androstene-3β,17β-diol-16-one (16-ketoandrostenediol)
h	31.47	649	258	473 (26)	418 (37)	244 (67)	143 (38)	129 (59)	5-pregnene-3β,11β,17α-triol-20-one
i	32.15	561	474	432 (7)	384 (13)	244 (49)	156 (53)	129 (53)	5-pregnene-3β,16α-diol-20-one (16α-hydroxypregnenolone)
j	32.85	649	117	474 (5)	227 (7)	213 (7)	156 (8)	129 (15)	
k	33.47	649	117	532 (6)	345 (8)	254 (6)	147 (26)	129 (16)	a 5-pregnenetriolone

* Figure reference, g.c. and m.s. conditions as noted in Figure 4.7. ** syn/anti isomers of oxime derivative.



Scheme 4.5.

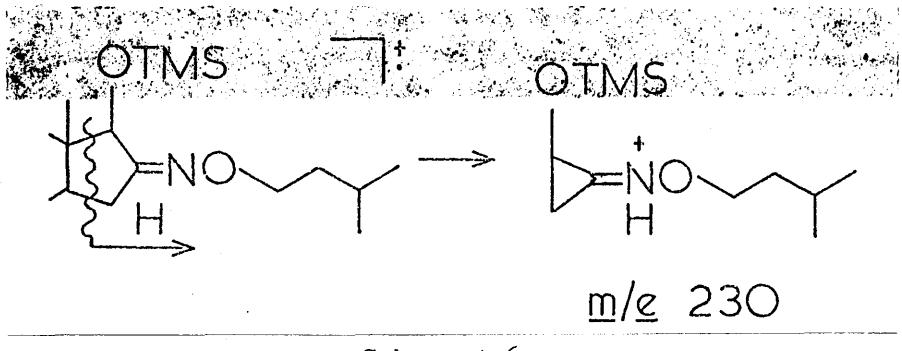
The observation of pregnenetriols in this g.c. region by other workers supports this.[132,450,473]

The first ketosteroid (d) appears as two peaks separated by about 15 index units. These arise from the syn- and anti-isomers of the alkyloxime derivative. The presence of this isomerism for some steroid derivatives has already been discussed. The odd mass number molecular ion shows prominently with a base peak of m/e 129, implying a Δ^5-3-OTMS structure. Cleavage of the N-O bond of the isopentyloxime results in an ion at m/e 446, $(M-87)^+$ and this, combined with m/e 356 $(M-87-90)^+$ and m/e 266 $(M-87-90-90)^+$, confirms the derivative as an isopentyloxime bis-trimethylsilyl ether. A fragmentation across ring D produces an ion of m/e 145, containing the oxime oxygen, nitrogen, C(16) and C(17) with its OTMS. Comparison with g.c. and m.s. data of the authentic compound confirms the parent steroid as 16α-hydroxydehydroepiandrosterone.[482]

Peak (e) at MU = 30.27 corresponds closely with an androstene-triolone reported by Baillie[474] as its iPO-TMS derivative and putatively assigned as 5-androstene-3β,11β,16-triol-17-one. Confirmatory g.c. and m.s. data were not available, nor do any of the androstenetriolones described by Shackleton[441] offer correlations.

Peak (f) at MU = 30.55 is immediately identifiable as cholesterol TMS ether.

The peak (g) at MU = 30.76 has some mass spectral features similar to those previously discussed: m/e 446 $(M-87)^+$, m/e 356 $(M-87-90)^+$, m/e 266 $(M-87-90-90)^+$, and m/e 129. A prominent ion at m/e 230 provides evidence for a 16-keto-17-hydroxy arrangement in ring D (Scheme 4.6.):



Scheme 4.6.

Similar ions retaining the keto or alkyloximino group appear in the spectra of other alkyloxime TMS ether derivatives of this compound (16-one bis-TMS ether m/e 145, 16-methyloxime bis-TMS ether m/e 174, 16-sec-butyloxime bis-TMS ether m/e 216)[474] and provide further evidence of this genesis. This data and retention index correlations support the identification of the parent compound as 16-keto-androstene-3β,17β-triol.

A prominent peak at MU = 31.47 (h) is indicated to be a pregnene-triolone isopentyloxime trimethylsilyl ether derivative by the mass of the molecular ion, m/e 649. This is further confirmed by an ion at m/e 472 $(M-87-90)^+$, while m/e 129 indicates a Δ^5-3-OTMS structure. The base peak, m/e 258 and a fragment ion m/e 244 are consistent with a 20-ketosteroid bearing a trimethylsilyloxy substituent in the region C(15)-C(21) and C(16)-C(21) respectively. These features together with comparison of g.c. data with published results permit the structure of the parent steroid to be assigned to 5-pregnene-3β,11β,17α-triol-20-one.[474]

The ion at m/e 244 from the compound of MU = 3215 (i) is also consistent with a 20-ketosteroid containing a trimethylsilyloxy substituent in the region C(16)-C(21) (i.e. in comparison with the simple 20-ketosteroid methyloxime: m/e 100 + 56 + 88). The molecular ion and the ion of m/e 129 support the Δ^5-3-OTMS structure and the identity is confirmed by comparison with the authentic 16α-hydroxypregnenolone derivative.[474]

The final prominent peaks, (j) and (k) at MU = 32.85 and 33.47 respectively, are indicated to be 5-pregnenetriolones by their molecular weights of m/e 649, prominent ions at m/e 129 (Δ^5-3-OTMS) and the base peak of m/e 117 whose significance has been discussed earlier.

The g.c.-m.s. data (Table 4.8.) confirm the predominance of metabolites with the 3β-hydroxy-Δ^5-structure in the androstane and pregnane series which are characteristic of the newborn period. The major component is 16α-hydroxypregnenolone. The co-occurrence of the three isomeric androstenediolones, namely 16α-hydroxy-DHA, 16β-hydroxy-DHA and 16-ketoandrostenediol, is a regular feature of newborn urinary steroids. The occurrence of syn/anti isomerism for the oxime derivatives of 16α-hydroxy-DHA[482] is a complicating feature in the assignment of peaks. The role of 16α-hydroxy-DHA in estriol biosynthesis is well established[48] whereas an explanation for the presence of the 16β isomer is not apparent. Furthermore, 16β-hydroxy-DHA has been shown to undergo spontaneous isomerisation to 16-ketoandrostenediol. In view of this with a prominent peak assigned to 16-ketoandrostendiol (g) the assignment of (d) as syn/anti isomers of 16α-hydroxy-DHA, rather than the

16α/16β isomer pair, is tentative but seems the most likely prospect. More thorough examination is warranted.

The advantages of higher chromatographic resolution are demonstrated here for the major constituents. The occurrence of mixed peaks persists (e.g. peak (e)). The application of higher efficiency glass open tubular columns with various group separations provided by different alkyloxime derivatives should be effective in resolving the remaining inconsistencies and extending the investigations to the quantitatively less prominent constituents.

Appendix A

Retention indices (I) and retention index increments (dI) for some sterols as acetate or trimethylsilyl ether derivatives.

The retention indices of the sterol acetates were calculated from relative retention times (RRT) recorded by Patterson.[399] This was possible because of the inclusion of RR^T values for bracketing n-alkanes. These data are supplemented by further RRT data converted to retention indices by internal standardisation. In this method two or more compounds common to both sets of data are chosen as bracketing relay standards to obtain by interpolation the retention indices of the remaining. This type of calculation tends to disguise differences induced by differing operating conditions. Conditions very similar to those of Patterson were employed in three other sources. These together provide a remarkably self-consistent tabulation. In a separate column (Table A.1.) retention indices calculated from data from other sources are included. While these are somewhat less relaible in the context of the whole they are included for useful internal correlations.

Correlation was undertaken (Table A.2.) and retention index increments (dI) extracted from these data (Tables A.3. and A.4.). A small number of increments for sterol TMS ethers on OV-17 by Middleditch[371] are also included for reference (Table A.5.).

Table A.1. Retention indices of some sterols as acetate derivatives.

Sterol acetate	Retention index $I^{SE-30}_{244°C}$[a]		
5β-Cholest-7-en-3β-ol (7-coprostenol)	3117		
5β-Cholestan-3β-ol (coprostanol)	3117	(3105)[i]	
5β-Cholest-8(14)-en-3β-ol	3117		
5,Z-22-Cholestadien-3β-ol (cis-22-dehydrocholesterol)	3122		
5β-Cholestan-3α-ol (epicoprostanol)		(3135)[i]	
5α-Cholestan-3α-ol (epicholestanol)	3138		
5,E-22-Cholestadien-3β-ol (trans-22-dehydrocholesterol)	3138		
5,22,24-Cholestatrien-3β-ol	3150		
5,7,22-Cholestatrien-3β-ol	3168		
5-Cholesten-3β-ol (cholesterol)	3172	(3172)[g]	(3200)[k]
5α-Cholestan-3β-ol (cholestanol)	3183	(3183)[g] (3210)[k]	(3158)[i]
5α-Cholesta-8(9),14-dien-3β-ol	3183		
14α-Methyl-5α-cholest-8(9)-en-3β-ol	3186		
5α-Cholest-8(9)-en-3β-ol	3190		
5,25-Cholestadien-3β-ol	3197		
5,24-Cholestadien-3β-ol (desmosterol)	3203	(3207)[f]	(3200)[k]
5,7-Cholestadien-3β-ol	3203		
5α-Ergosta-8(14),22-dien-3β-ol	3203		
5,22-Ergostadien-3β-ol (brassicasterol)	3213	(3217)[g]	(3240)[k]
5α-Cholest-7-en-3β-ol	3213		
5α-Ergosta-7,14,22-trien-3β-ol	3217		
5α-Cholesta-8(9),24-dien-3β-ol (zymosterol)	3217	(3217)[d]	
5α-Ergosta-8(9),14,22-trien-3β-ol	3220		
5,8(9),22-Ergostatrien-3β-ol		(3220)[c]	(3233)[g]
14α-Methyl-5α-ergosta-8(9),22-dien-3β-ol	3227		
14α-Methyl-9β,19-cyclo-5α-cholestan-3β-ol (pollinastanol)		(3227)[b]	
5α-Ergosta-8(9),22-dien-3β-ol	3229		

14α-Methyl-5α-cholest-7-en-3β-ol	3229	
5α-Ergosta-7,9(11),22-trien-3β-ol	3233	
5,7,E-22-Ergostatrien-3β-ol (ergosterol)	3245	(3245)[b,c,d] (3251)[g] (3257)[i]
5α-Cholesta-7,24-dien-3β-ol		(3245)[d]
5α-Ergosta-7,22-dien-3β-ol	3254	(3254)[b,c,d]
5,24(28)-Ergostadien-3β-ol (24-methylenecholesterol)	3257	(3300)[k]
4α-Methyl-5α-cholest-7-en-3β-ol (lophenol)	3260	
5α-Ergost-8(14)-en-3β-ol	3262	(3262)[b]
14α-Methyl-5α-ergosta-7,22-dien-3β-ol	3265	
(24R)-24-Methyl-5-cholesten-3β-ol (campesterol)	3268	(3300)[k]
5-Ergosten-3β-ol	3268	
14α-Methyl-5α-ergosta-8(9),24(28)-dien-3β-ol		(3268)[i]
4,4-Dimethyl-5α-cholest-7-en-3β-ol		(3277)[i]
5α-Ergosta-8(9),24(28)-dien-3β-ol	3277	
5α-Ergosta-8(9),14-dien-3β-ol	3277	(3277)[b]
5,8(9)-Ergostadien-3β-ol		(3277)[c]
(24R)-24-Methyl-5α-cholestan-3β-ol (campestanol)	3279	(3265)[g]
14α-Methyl-5α-ergost-8(9)-en-3β-ol	3280	(3280)[b]
4α,14α-Dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (31-nor-cycloartanol)	3282	
5α-Ergost-8(9)-en-3β-ol	3285	(3285)[b]
5α-Lanosta-7,9(11)-dien-3β-ol (dihydroagnosterol)		(3290)[i]
5α-Lanost-8(9)-en-3β-ol (24,25-dihydrolanosterol)	3298	
4α-Methyl-5α-cholesta-7,24-dien-3β-ol		(3298)[i]
5,7-Ergostadien-3β-ol	3301	(3301)[b,c]
5,22-Stigmastadien-3β-ol (stigmasterol)	3301	(3330)[g] (3311)[i] (3220)[k]
(24R)-24-Ethyl-5,22-cholestadien-3β-ol (poriferasterol)	3301	
5α-Ergosta-7,24(28)-dien-3β-ol (episterol)	3301	(3298)[d]
14α-Methyl-9β,19-cyclo-5α-ergosta-24(28)-en-3β-ol (24-methylenepollinastanol)	3311	
5α-Ergost-7-en-3β-ol	3311	(3311)[c,d] (3318)[g]
4α,14α-Dimethyl-5α-ergosta-8(9),24(28)-dien-3β-ol (obtusifoliol)	3313	

4α,14α-Dimethyl-5α-ergost-8(9)-en-3β-ol (24-dihydroobtusifoliol)	3321		
4α-Methyl-5α-ergosta-8(9),14-dien-3β-ol	3321		
14α-Methyl-9β,19-cyclo-5α-ergostan-3β-ol (24-methylpollinastanol)	3323	(3323)[b]	
5α-Lanosta-7,9(11),24-trien-3β-ol		(3323)[i]	
5α-Ergosta-7,24(28)-dien-3β-ol		(3326)[g]	
14α-Methyl-5α-ergost-7-en-3β-ol	3326		
5α-Lanosta-8(9),24-dien-3β-ol (lanosterol)	3330	(3333)[c]	(3342)[i]
5,7,22-Stigmastatrien-3β-ol	3330		
4α-Methyl-5α-ergost-8(9)-en-3β-ol	3333		
5,25-Stigmastadien-3β-ol	3333		
5α-Stigmasta-7,22,25-trien-3β-ol	3337		
5α-Stigmasta-7,22-dien-3β-ol (α-spinasterol)	3340		
(24R)-24-Ethyl-5α-cholesta-7,22-dien-3β-ol (chondrillasterol)	3340		
4,4-Dimethyl-5α-cholesta-8(9),24-dien-3β-ol		(3342)[i]	
5α-Lanost-8(9)-en-3β-ol		(3344)[i]	
4α-Methyl-5α-ergosta-7,24(28)-dien-3β-ol	3344		
9β,19-Cyclo-5α-lanostan-3β-ol (cycloartanol)	3347		
5α-Stigmast-8(14)-en-3β-ol	3347		
5-Stigmasten-3β-ol (sitosterol)	3351	(3330)[g]	(3380)[k]
(24S)-24-Ethyl-5-cholesten-3β-ol	3351		
5,E-24(28)-Stigmastadien-3β-ol (fucosterol)	3351		
4α-Methyl-5α-ergosta-7,24(28)-dien-3β-ol (24-methylenelophenol)		(3353)[h]	
5α-Stigmasta-8(9),14-dien-3β-ol	3358		
5α-Stigmastan-3β-ol	3360	(3340)[g]	
(22R,23R,24R)-22,23-Methylene-24-methyl-5-cholesten-3β-ol (23-demethylgorgosterol)	3360		
14α-Methyl-5α-stigmast-8(9)-en-3β-ol	3360		
5,Z-24(28)-Stigmastadien-3β-ol (28-isofucosterol,avenasterol)	3364		
4α,14α-Dimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (cycloeucalenol)	3367		
5α-Stigmast-8(9)-en-3β-ol	3367		
5α-Lanost-7-en-3β-ol		(3369)[i]	
5α-Stigmasta-8(9),14,24(28)-trien-3β-ol	3369		

5α-Stigmasta-7,25-dien-3β-ol	3373		
9β,19-Cyclo-5α-lanost-25-en-3β-ol (cycloartenol)	3377	(3372)[b]	(3371)[e]
5,7-Stigmastadien-3β-ol	3384		
5α-Stigmast-7-en-3β-ol	3394	(3390)[g]	(3410)[k]
(24S)-24-Ethyl-5α-cholest-7-en-3β-ol (22-dihydrochondrillasterol)	3394	(3370)[k]	
14α-Methyl-5α-cholest-8(9)-ene-3β,6α-diol (macdougallin)	3396		
4α-Methyl-5α-stigmasta-8(9),14-dien-3β-ol	3402		
4α,14α-Dimethyl-5α-stigmast-8(9)-en-3β-ol	3404		
5α-Stigmasta-7,Z-24(28)-dien-3β-ol (7-avenasterol)	3404		
4α-Methyl-5α-stigmast-8(9)-en-3β-ol	3411		
5α-Cholest-8(9)-ene-3β,6α-diol	3411		
4,4,14α-Trimethyl-9β,19-cyclo-5α-ergost-25-en-3β-ol (cyclolaudenol)	3415	(3415)[b]	
4,4,14α-Trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol (24-methylenecycloartanol)	3423	(3430)[e]	
4,4,14α-trimethyl-9β,19-cyclo-5α-ergostan-3β-ol (24-methylcycloartanol)	3437		
4,4-Dimethyl-5α-cholesta-7,24-dien-3β-ol		(3437)[i]	
4α-Methyl-5α-stigmasta-7,Z-24(28)-dien-3β-ol (citrostadienol, 24-ethylidenelophenol)	3449	(3458)[h] (3465)[k]	
(22R, 23R, 24R)-22,23-Methylene-23,24-dimethyl-5-cholesten-3β-ol (gorgosterol)	3465		
5,28-Stigmastadiene-3β,24ξ-diol (saringosterol)	3508		

a) Retention indices calculated from acetate and n-alkane RRT data according to G.W. Patterson, Anal. Chem., 43 (1971) 1165-1170. G.c. conditions: 3% SE-30 on Gas Chrom Q, 244°C, Argon 150ml/min.

Calculated by relay with reference to (a) from RRT data:

b) J.T. Chan, G.W. Patterson, S.R. Dutky and C.F. Cohen, Plant Physiol., 53 (1974) 244-249. G.c. conditions as in (a).

c) G.W. Patterson, M.J. Thompson and S.R. Dutky, Phytochem., 13 (1974) 191-194. G.c. conditions as in (a).

d) L.W. Parks, C. Anding and G. Ourisson, Eur. J. Biochem., 43 (1974) 451-458. G.c. conditions: 1% SE-30, 270°C, N_2 30ml/min.

e) G.F. Gibbons, L.J. Goad, T.W. Goodwin and W.R. Nes, J. Biol. Chem., 246 (1971) 3967-3976. G.c. conditions: 1% SE-30, 250°C.

f) J.A. Svoboda and M.J. Thompson, J. Lipid Res., 8 (1967) 152-154. G.c. conditions: 0.75% SE-30, 270°C.

g) Z.A. Wojciechowski, L.J. Goad and T.W. Goodwin, Phytochem., 12 (1973) 1433-1436. G.c. conditions: 3% SE-30, 270°C.

h) L.J. Goad and T.W. Goodwin, Eur. J. Biochem., 1 (1967) 357-362. G.c. conditions: 1% SE-30, 225°C, N_2 40ml/min.

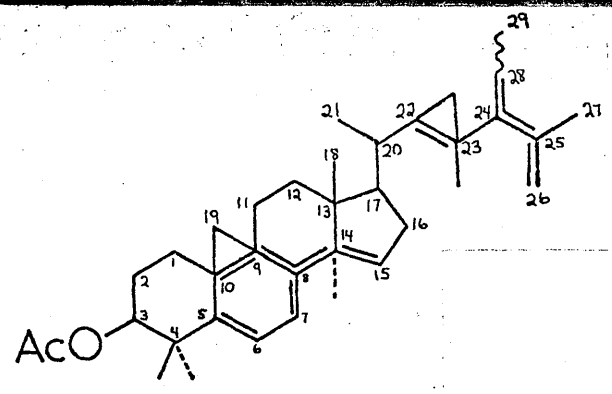
i) A.G. Smith, I. Rubinstein and L.J. Goad, Biochem. J., 135 (1973) 443-445. G.c. conditions: 1% SE-30, 250°C.

j) C.J.W. Brooks and L. Hanaineh, Biochem. J., 87 (1963) 151-160. G.c. conditions: 1% SE-30, 200°C.

k) Retention indices according to B.A. Knights, in Modern Methods of Steroid Analysis (E. Heftmann ed.) Academic Press, New York, 1973 pp. 103-138. G.c. conditions: 1% OV-101, 255°C.

Table A.2. Correlation chart for relating structure to retention index of sterol acetate derivatives on methyl-silicone stationary phase.*

Sidechain	Nucleus																			
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
1	3183	3172	3190	3213	-	3203	3183	-	-	-	-	3260	3186	3229	3227	-	-	3282	3247	3298
2	-	3122 (cis) 3138 (trans)	-	-	3203	3168	-	-	-	-	-	-	3227	-	-	-	-	-	-	-
3	-	3203	3217	3245	-	-	-	-	-	-	-	-	-	-	-	-	-	3367	3377	3330
4	-	3197	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	3150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	3279	3268	3285	3311	3262	3301	3277	-	-	3277	3333	-	3280	3326	3323	3321	3321	-	3437	-
7	-	3213	3229	3254	3203	3245	3220	3217	3233	3220	-	-	3227	3265	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3415	-
9	-	3257	3277	3301	-	-	-	-	-	-	-	3344	3268	-	3311	-	3313	-	3423	-
10	3360	3351	3367	3394	3347	3384	3358	-	-	-	3411	-	3360	-	-	3402	3404	-	-	-
11	-	3301	-	3340	-	3330	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	3333	-	3373	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	3351	-	-	-	-	3369	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	3364	-	3404	-	-	-	-	-	-	-	3449	-	-	-	-	-	-	-	-
15	-	-	-	3337	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	3360	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	3465	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-



COMPOSITE FORMULA

Nucleus

A	C19-5α
B	C19-Δ^{5}
C	C19-5α-$\Delta^{8(9)}$
D	C19-5α-Δ^{7}
E	C19-5α-$\Delta^{8(14)}$
F	C19-$\Delta^{5,7}$
G	C19-$\Delta^{8(9),14}$-5α
H	C19-$\Delta^{7,(14)}$-5α
I	C19-$\Delta^{7,9(10)}$-5α
J	C19-$\Delta^{5,8(9)}$
K	C20-4α-methyl-5α-$\Delta^{8(9)}$
L	C20-4α-methyl-5α-Δ^{7}
M	C20-14α-methyl-5α-$\Delta^{8(9)}$
N	C20-14α-methyl-5α-Δ^{7}
O	C20-14α-methyl-9β,19-cyclo-5α
P	C20-4α-methyl-5α-$\Delta^{8(9),14}$
Q	C21-4α,14α-dimethyl-5α-$\Delta^{8(9)}$
R	C21-4α,14α-dimethyl-9β,19-cyclo-5α
S	C22-4,4,14α-trimethyl-9β,19-cyclo-5α
T	C22-4,4,14α-trimethyl-5α-$\Delta^{8(9)}$

Sidechain

1	C8
2	C8-Δ^{22}
3	C8-$\Delta^{24(25)}$
4	C8-$\Delta^{25(26)}$
5	C8-$\Delta^{22,24(25)}$
6	C9-24-methyl
7	C9-24-methyl-Δ^{22}
8	C9-24-methyl-$\Delta^{24(25)}$
9	C9-24-methylene-$\Delta^{24(28)}$
10	C10-24-ethyl
11	C10-24-ethyl-Δ^{22}
12	C10-24-ethyl-$\Delta^{24(25)}$
13	C10-24-ethylidene-$\Delta^{24(28)}$-E
14	C10-24-ethylidene-$\Delta^{24(28)}$-Z
15	C10-24-ethyl-$\Delta^{22,24(25)}$
16	C10-24-methyl-22,23-methylene
17	C11-23,24-dimethyl-22,23-methylene

* Data extracted from calculated retention indices of sterol acetates (Table A.1.) as per sources noted there: a,b,c,d. Chromatographic conditions: 3% SE-30 on Gas Chrom Q, 244°C, Argon 150ml/min.

Table A.3. Retention index increments (dI) for double bond and steric transformations in sterol acetate derivatives on methyl silicone stationary phase (SE-30).

Double bonds	Sterols compared	Carbon atoms	dI
Nuclear double bond increments			
Δ^5	$\Delta^5/5\alpha$-stanol	C27(C8)	-11
		C28(C9)	-11
		C29(C10)	-11
Δ^7	$\Delta^7/5\alpha$-stanol	C27(C8)	+30
		C28(C9)	+32
		C29(C10)	+34
$\Delta^{8(9)}$	$\Delta^{8(9)}/5\alpha$-stanol	C27(C8)	+7
		C28(C9)	+6
		C29(C10)	+7
$\Delta^{8(14)}$	$\Delta^{8(14)}/5\alpha$-stanol	C28(C9)	-17
		C28(C10)	-13
$\Delta^{5,7}$	$\Delta^{5,7}/5\alpha$-stanol	C27(C8)	+20
		C28(C9)	+22
		C29(C10)	+24
$\Delta^{5,8(9)}$	$\Delta^{5,8(9)}/5\alpha$-stanol	C28(C9)	-2
$\Delta^{8(9),14}$	$\Delta^{8(9),14}/5\alpha$-stanol	C27(C8)	0
		C28(C9)	-2
		C30(C10)	-2
Nuclear steric increments			
$5\alpha \longrightarrow 5\beta$	cholestanol/coprostanol	C27(C9)	-66
$3\alpha \longrightarrow 3\beta$	epicholestanol/cholestanol	C27(C9)	-45
Side-chain double bond increments			
Δ^{22}(cis)	$\Delta^{5,22}/\Delta^5$	C27(C8)	-50
Δ^{22}(trans)	$\Delta^{5,22}/\Delta^5$	C27(C8)	-34
		C28(C9)	-55
		C29(C10)	-50
	$\Delta^{5,22,24(25)}/\Delta^{5,24(25)}$	C27(C8)	-47
	$\Delta^{5,7,22}/\Delta^{5,7}$	C27(C8)	-35
		C28(C9)	-56
		C29(C10)	-54

Table A.3. (cont'd.)

	$\Delta^{7,22}/\Delta^{7}$	C28(C9)	-43
		C29(C10)	-54
		C29(C9)	-39
	$\Delta^{8(9),22}/\Delta^{8(9)}$	C28(C9)	-57
		C29(C8)	-41
		C30(C9)	-43
	$\Delta^{8(9),14,22}/\Delta^{8(9),14}$	C28(C9)	-57
	$\Delta^{8(14),22}/\Delta^{8(14)}$	C28(C9)	-59
$\Delta^{24(25)}$	$\Delta^{24(25)}/5\alpha$-stanol	C30(C8)	+30
	$\Delta^{5,24(25)}/\Delta^{5}$	C27(C8)	+31
	$\Delta^{7,24(25)}/\Delta^{7}$	C27(C8)	+32
	$\Delta^{8(9),24(25)}/\Delta^{8(9)}$	C27(C8)	+27
		C30(C8)	+32
	$\Delta^{5,22,24(25)}/\Delta^{5,22}$	C27(C8)	+8
$\Delta^{24(28)}$	$\Delta^{24(28)}/5\alpha$-stanol	C29(C9)	-12
		C31(C9)	-14
	$\Delta^{5,24(28)}/\Delta^{5}$	C28(C9)	-11
	$\Delta^{7,24(28)}/\Delta^{7}$	C28(C9)	-13
	$\Delta^{8(9),24(28)}/\Delta^{8(9)}$	C28(C9)	-8
		C29(C9)	-12
		C30(C9)	-8

Side-chain steric increments

Δ^{22}(cis)/Δ^{22}(trans)	C27(C8)	+16
$\Delta^{24(28)}$-E/$\Delta^{24(28)}$-Z	C29(C10)	+13

Table A.4. Retention index increments (dI) for alkyl substituents in sterol acetate derivatives on methyl silicone stationary phase (SE-30).

Substituent	Sterols compared (Carbon atoms)	Double bonds	dI
24-methyl	27/28	$\Delta^{5,22}$	+91
		$\Delta^{5,7,22}$	+97
		Δ^{5}	+90
		$\Delta^{8(9)}$	+95
		Δ^{7}	+98
		$\Delta^{5,7}$	+98
		$\Delta^{8(9),14}$	+94
		5α-stanol	+96
	28/29	Δ^{7}	+94
		$\Delta^{8(9)}$	+94
	30/31	5α-stanol	+100
24-ethyl	27/29	$\Delta^{5,22}$	+179
		Δ^{5}	+179
		Δ^{7}	+187
		$\Delta^{8(9),14}$	+175
		$\Delta^{8(9)}$	+177
	28/30	$\Delta^{8(9)}$	+174
24-methyl/24-ethyl	28/29	$\Delta^{5,22}$	+88
		$\Delta^{7,22}$	+86
		$\Delta^{5,7,22}$	+85
		Δ^{5}	+83
		Δ^{7}	+83
		$\Delta^{5,7}$	+83
		$\Delta^{8,(9),14}$	+81
		5α-stanol	+81
		$\Delta^{8(9)}$	+80
	30/29	$\Delta^{8(9)}$	+82
4α-methyl	28/29	5α-stanol	+65
	29/30	$\Delta^{24(28)}$	+90
	28/29	$\Delta^{7,24(28)}$	+43
	28/29	$\Delta^{8(9)}$	+48
	30/31	$\Delta^{8(9)}$	+54
	29/30	$\Delta^{8(9),14}$	+44
	28/29	$\Delta^{8(9),14}$	+44
	27/28	Δ^{7}	+47
14α-methyl	28/29	$\Delta^{8(9)}$	-4
	29/30	$\Delta^{8(9)}$	-7
	28/29	$\Delta^{8(9),22}$	-2
	28/29	$\Delta^{8(9),24(28)}$	-9
	27/28	Δ^{7}	+15
	28/29	Δ^{7}	+15
	28/29	$\Delta^{7,22}$	+16
4,4-dimethyl/4α-methyl	30/29	5α-stanol	+35

Table A.5. Retention index increments for double bonds and alkyl substituents in sterol trimethylsilyl ethers on methyl-phenyl silicone stationary phase (OV-17).*

Structure	dI**
Δ^5	0
Δ^7	+65
$\Delta^{8(9)}$	+15
$\Delta^{8(14)}$	+15
$\Delta^{9(11)}$	+45
Δ^{22}	-50
Δ^{24}	+75
$\Delta^{24(28)}$	+15
4α-methyl	+60
4β-methyl	+35
14-methyl	-10
24-methyl	+100

* from B.S. Middleditch, Ph.D. Thesis, University of Glasgow, 1971.

** referred to 5α-cholestan-3β-ol $I^{OV-17}_{240^o} = 3230$

G.c. conditions: 12ft 1% OV-17 on 100-120mesh Gas Chrom Q, N_2 40ml/min, 240°C.

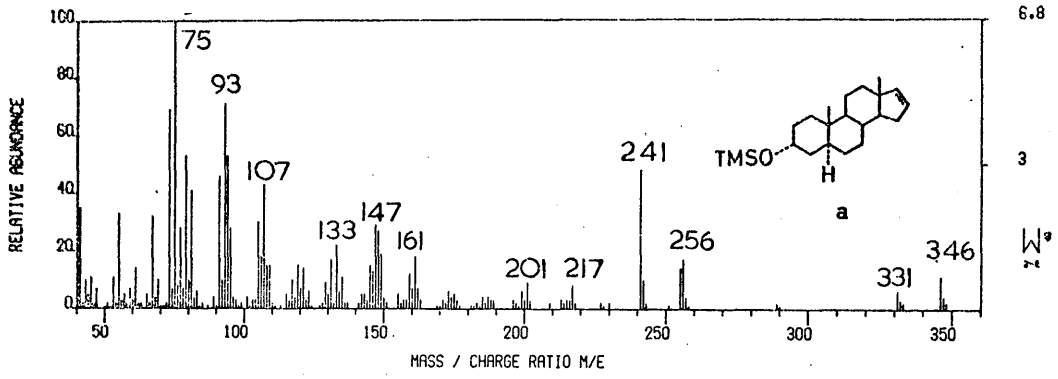
Appendix B

Mass spectra of some standard compounds

Mass spectra were obtained on the LKB 9000, source temperature and separator temperature 270°C, electron energy 70eV, emission current 60μA.

Sources of standard compounds and other relevant details are discussed in the appropriate portions of the text: Section 2.3.8., 2.4.2. and 3.3.2.

5ALPHA-ANDROST-16-EN-3ALPHA-OL TMS ETHER 74/859/I 70EV MW346



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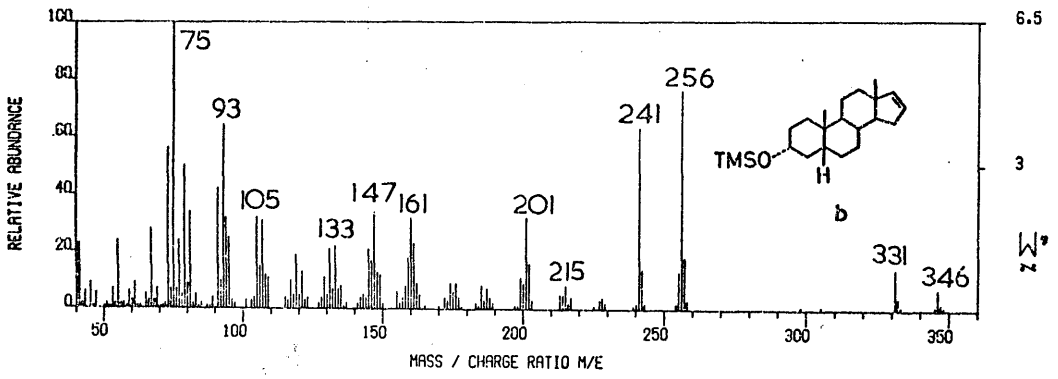
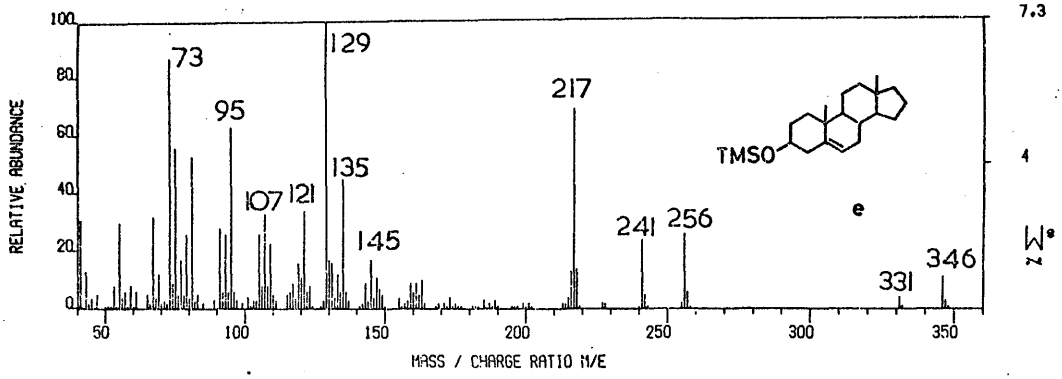
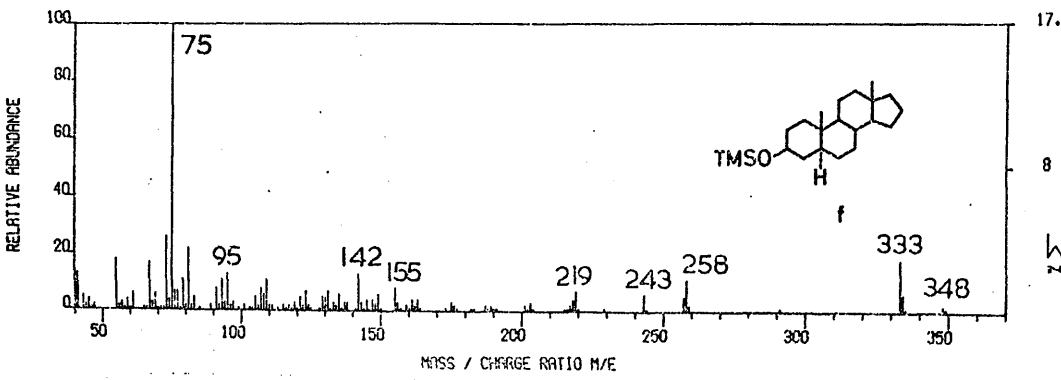


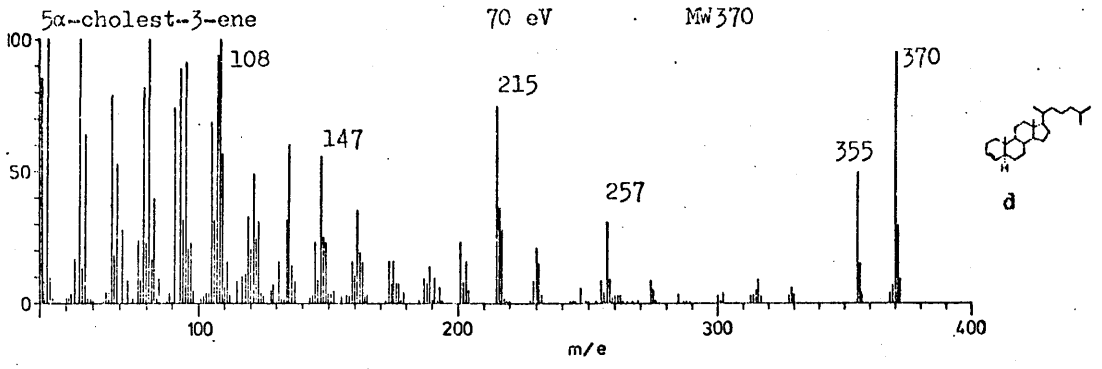
ANDROST-5-EN-3BETA-OL TMS ETHER 74/856/II 70 EV MW346

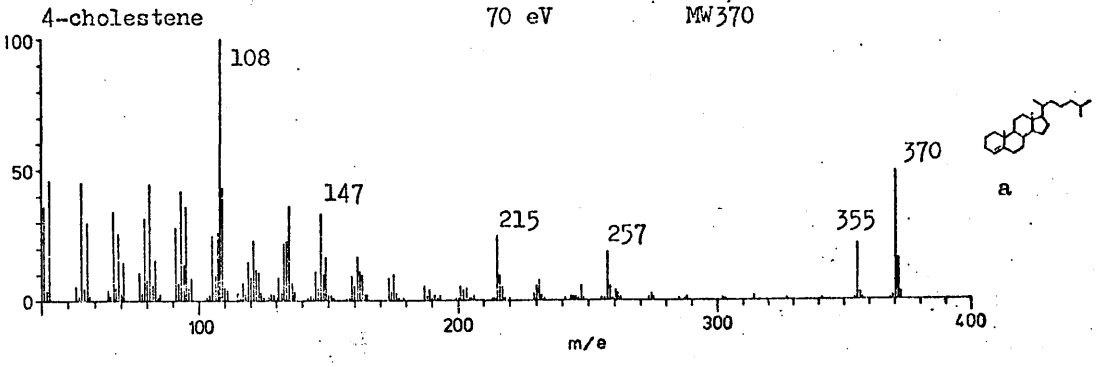


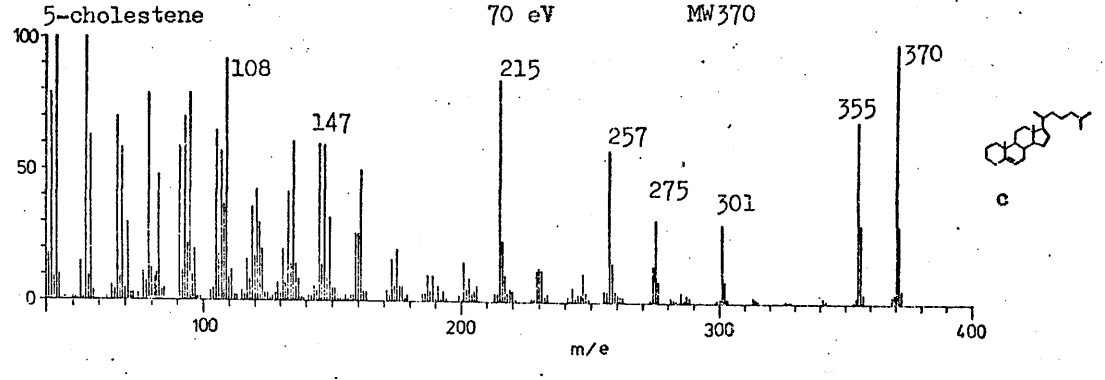
5ALPHA-ANDROSTAN-3BETA-OL TMS ETHER 74/855/I 70EV MW348

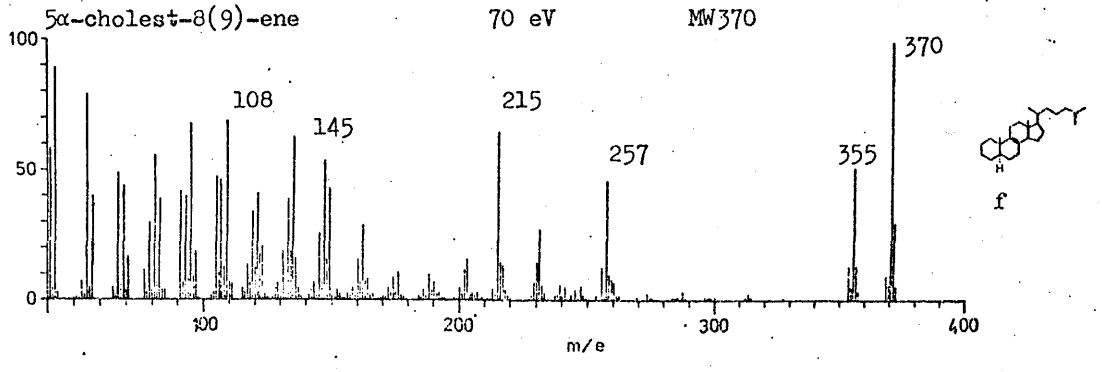


facing page 181.

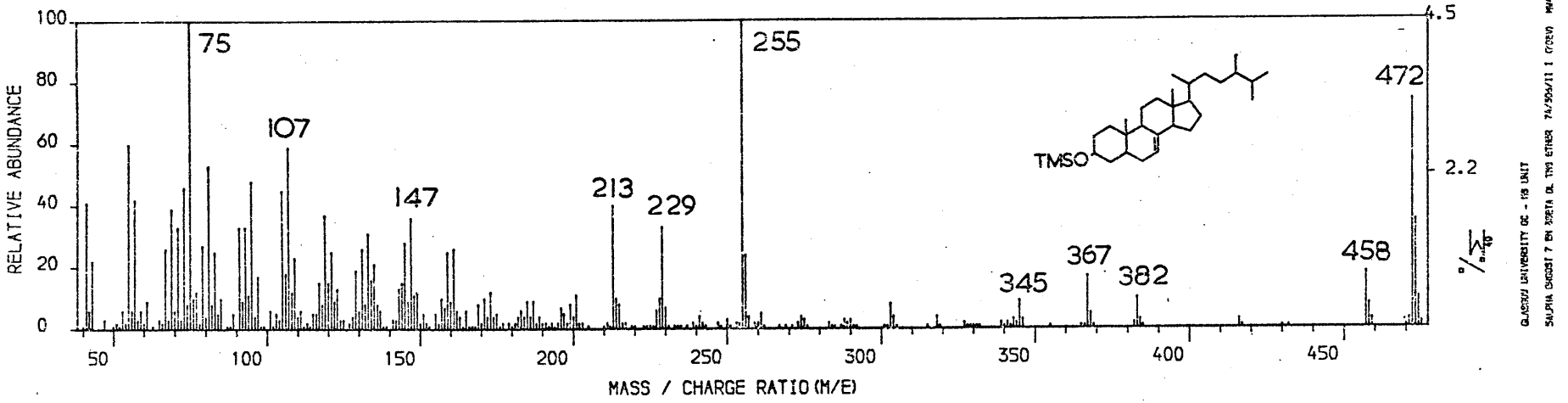




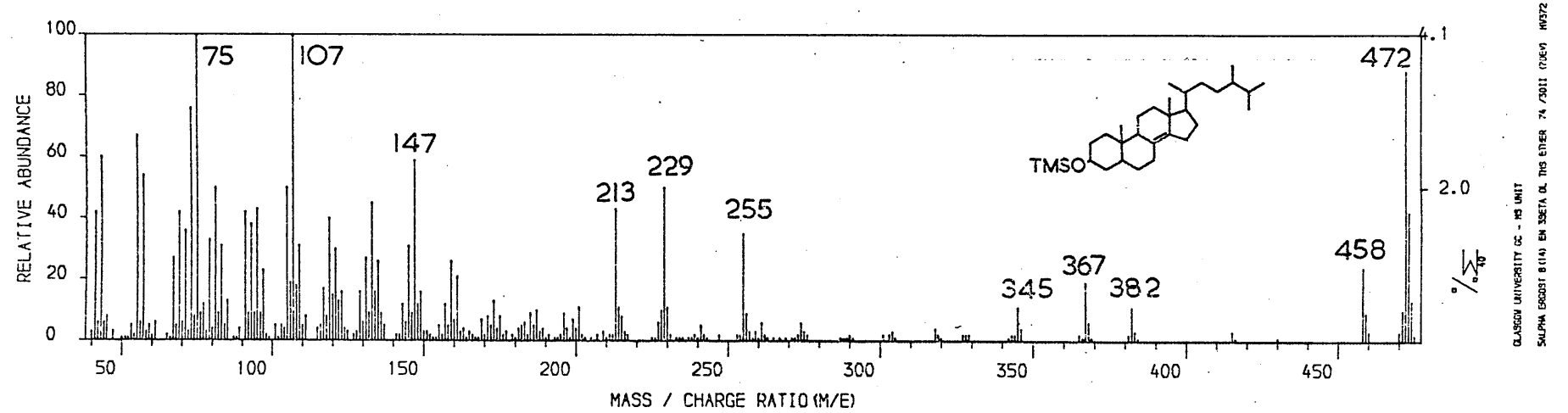




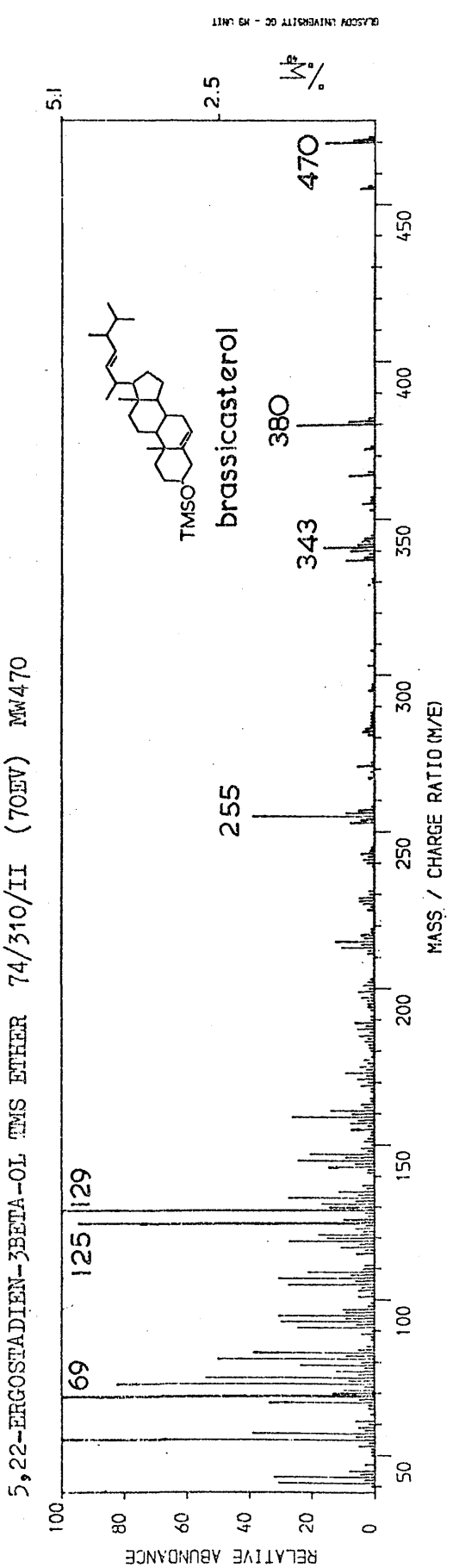
5ALPHA-ERGOST-7-EN-3BETA-OL TMS ETHER 74/306/II I (70EV) MW472

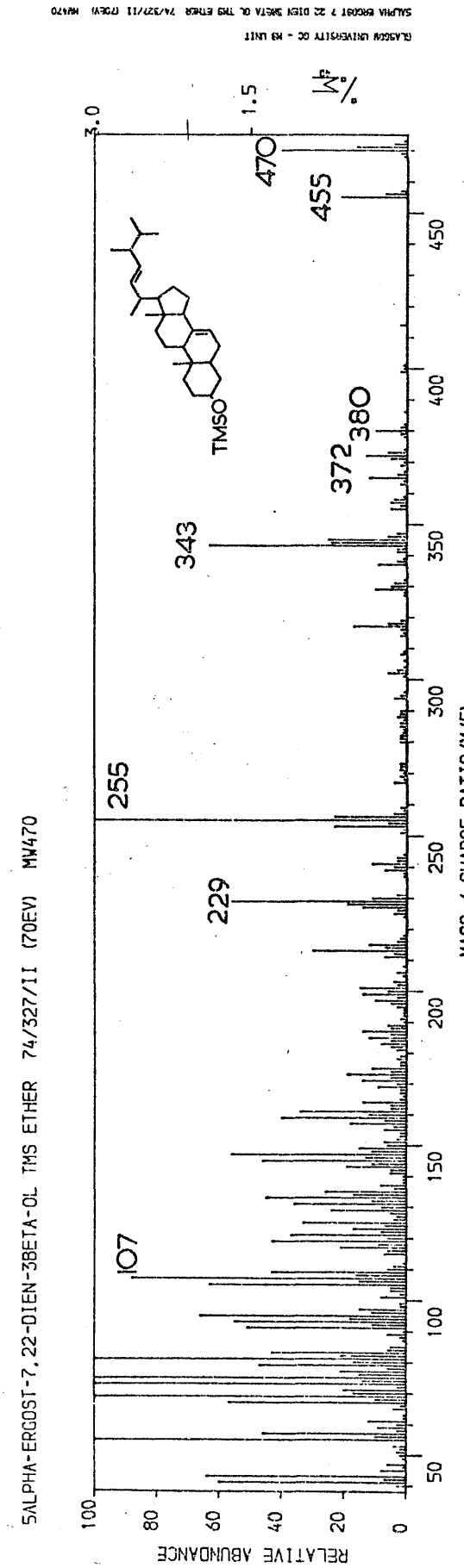


5ALPHA-ERGOST-8(14)-EN-3BETA-OL TMS ETHER 74 /30II (70EV) MW472

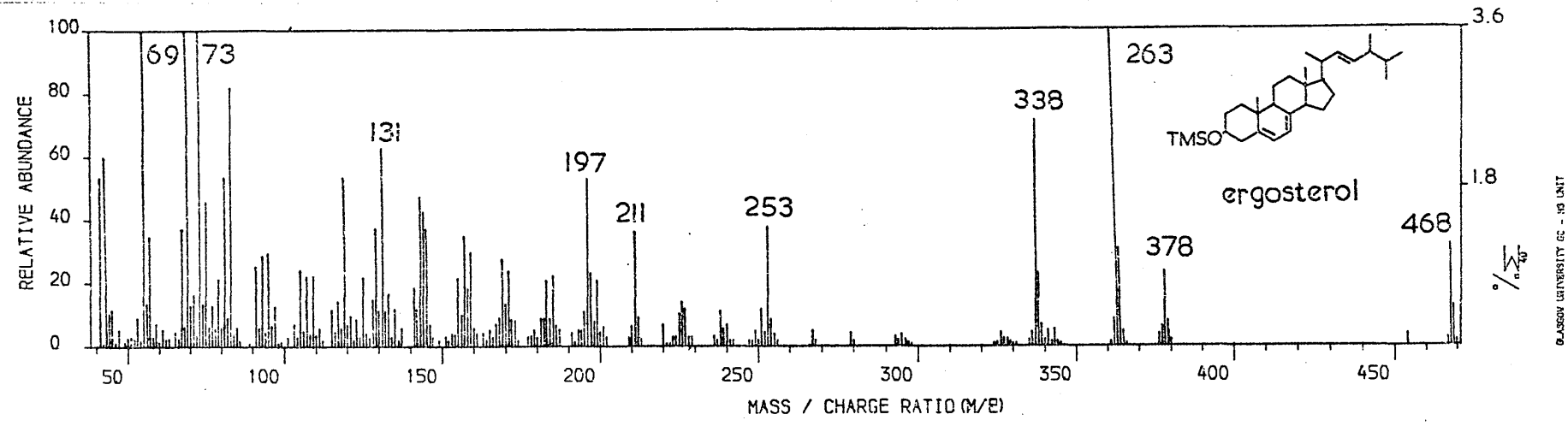


facing page 182.

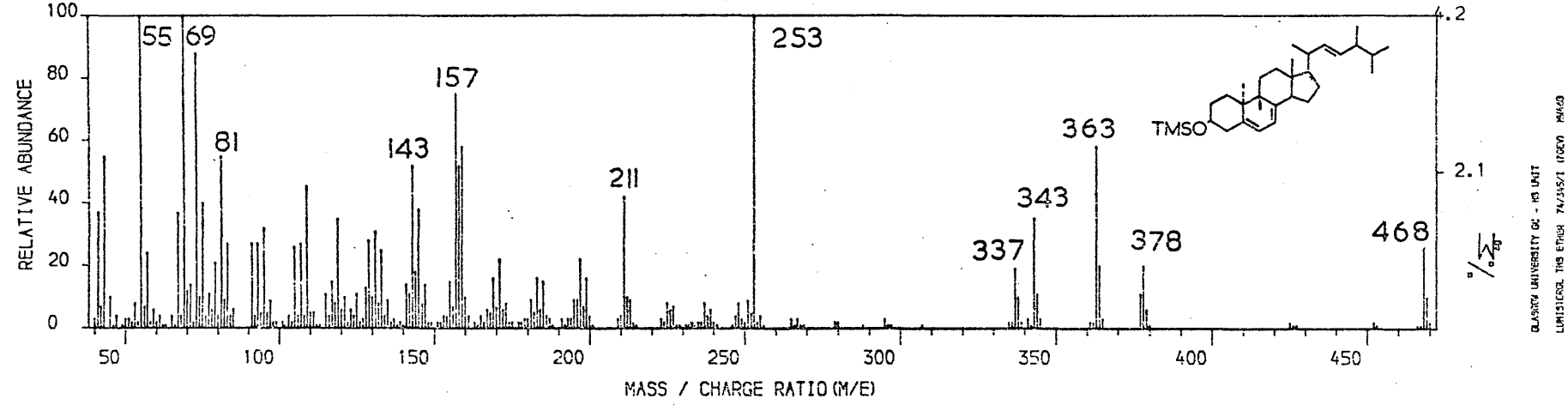




5,7,22-ERGOSTATRIEN-3BETA-OL TMS ETHER 74/328/II (70EV) MW468



LUMISTEROL TMS ETHER 74/345/I (70EV) MW468



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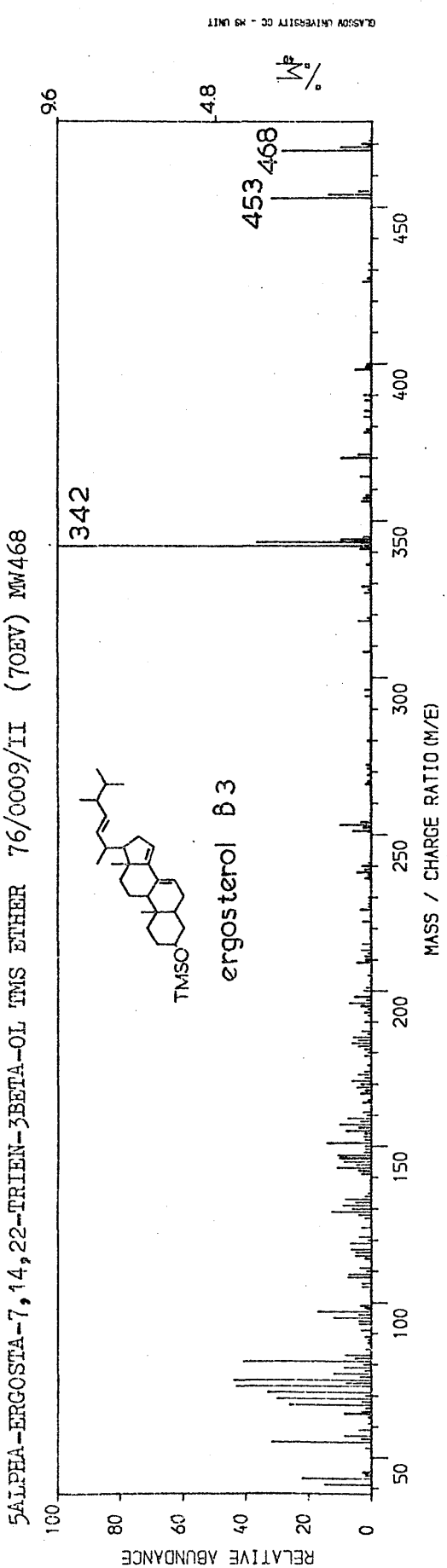

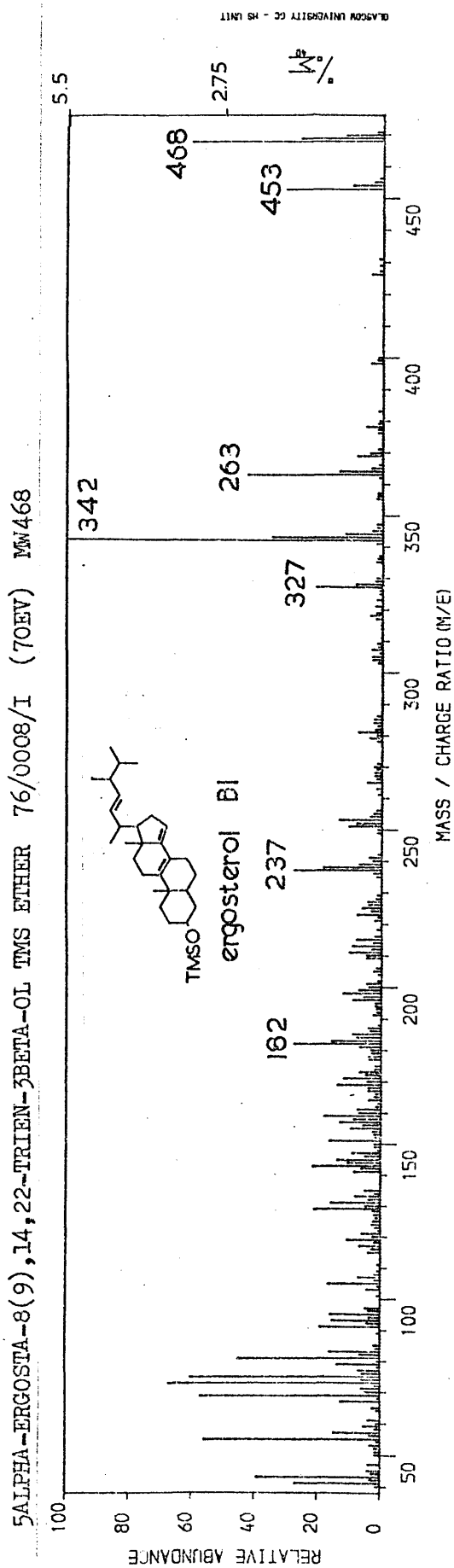

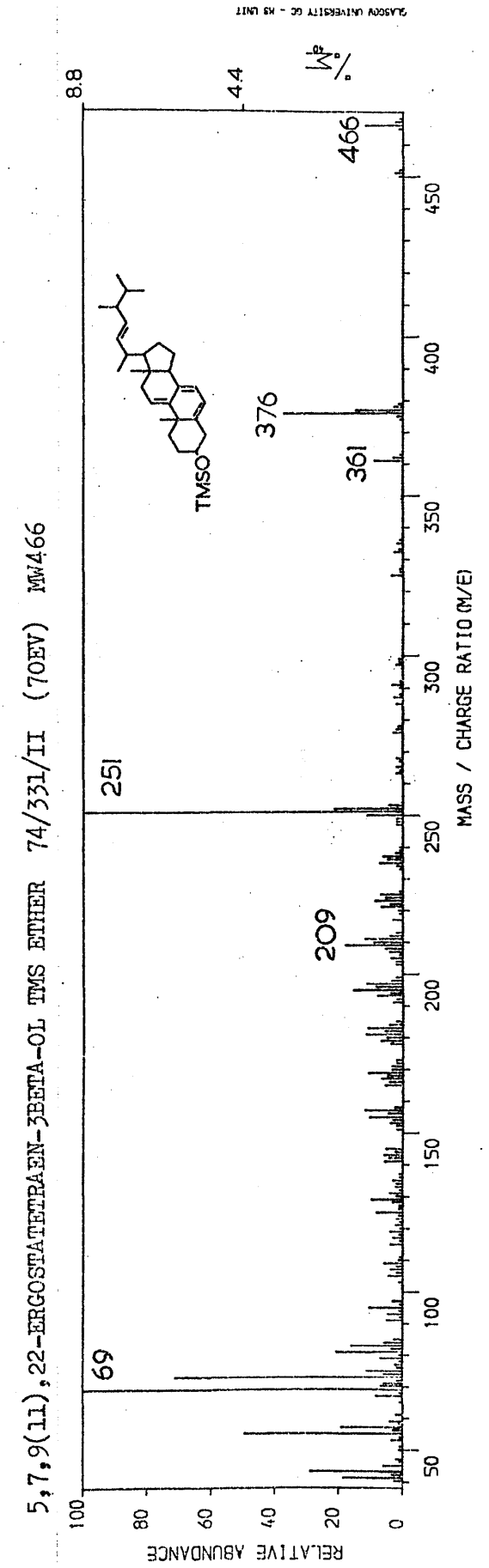
GLASGOW UNIVERSITY GC - MS UNIT
5,7,9(11),22-ERGOSTATETRAEN-3BETA-OL TMS ETHER 74/331/II (70EV) MW466
RELATIVE ABUNDANCE
MASS / CHARGE RATIO (M/E)
69
209
251
361
376
466
TMSO
8.8
4.4

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