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A Gas–Liquid-Chromatographic Procedure for Separating a Wide Range of Metabolites occurring in Urine or Tissue Extracts

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1. A gas–liquid-chromatographic procedure is described which permits separation and identification on the same chromatogram of a wide range of substances occurring in urine or tissue extracts. The method uses hydrogen flame ionization, which detects organic compounds whether free or conjugated with no requirement for specific reactive groups. 2. For chromatography, carboxyl groups are quantitatively converted into methyl esters or trimethylsilyl esters. Phenolic, alcoholic and potential enolic groups are converted into trimethylsilyl ethers. Separations are carried out on a 6ft. column of either 10% F-60 (a polysiloxane) or 1% F-60, temperature programming at 2°/min. being used over such part of the temperature range 30°–260° as is required. Propionyl derivatives of hydroxy compounds can also be used, but only on a non-quantitative basis. Derivatives and columns have been selected for optimum range of usefulness when large numbers of samples are examined by using automated gas chromatography. 3. The method is applicable to: fatty acids above butyric acid; di- and tri-carboxylic acids; hydroxy acids and keto acids; polyhydroxy and alicyclic compounds such as glycerol, inositol, quinic acid, shikimic acid, ascorbic acid and sugar alcohols; aromatic hydroxy and acidic compounds, both benzenoid and indolic; sesquiterpenes; steroids; glycine conjugates; mercapturic acids; glucuronides. It is not satisfactory for sulphate conjugates, iminazoles or polypeptides. 4. Methylene units provide an accurate and reproducible parameter for characterizing peak position. Methylene unit values are reported for a large variety of substances occurring in, or related to those occurring in, urine and tissue extracts. 5. The nature of derivatives was confirmed by combining gas chromatography with mass spectrometry. Combined gas chromatography–mass spectrometry gives a diagnostic tool of great power in the evaluation of metabolic patterns, and various uses are discussed.

Urine contains a vast number of substances representing intermediates and end-products of metabolic pathways of both endogenous and exogenous compounds. Many of these substances are of great interest in metabolic investigations, although quantitatively they may be only minor components, and satisfactory procedures have been developed for the separation of numerous physiologically significant metabolites in urine either as individual compounds or as groups of related compounds. However, changes in the amount of a single substance occurring in urine may be of much less significance than changes in the relative amounts of several substances which may be only distantly related chemically.

To meet our needs for metabolic studies a separative procedure was required which had higher sensitivity, specificity and resolution, and applicability to a wider range of types of compound, than any procedure described in the literature. Moreover we needed a procedure which permitted quantitative estimation as well as identification, and which was sufficiently simple to allow large numbers of analyses to be performed. To meet these requirements we turned to gas chromatography, and developed a procedure which offers many advantages for separating and estimating metabolites in urine and tissues. A hydrogen flame ionization-detection system is employed; this detects organic compounds whether free or conjugated, and the response is not dependent on the presence of specific reactive groups in the molecule. By using a series of straight-chain hydrocarbons as reference standards, substances

can be characterized chromatographically with a high degree of reproducibility in terms of their 'methylene unit' values. By attaching the gas chromatograph to a mass spectrometer, substances can also be characterized structurally. This paper describes and evaluates the gas-chromatographic behaviour of known substances of many chemical types occurring in, or related to those occurring in, urine or tissue extracts.

METHODS

Starting materials. Commercially available materials were used in most cases. Some methyl ethers of phenolic compounds which were not commercially available were prepared by prolonged treatment of a methanolic solution of the phenol with excess of ethereal diazomethane.

In addition we are grateful to the following for the gift of authentic compounds: Professor R. T. Williams (anisuric acid, 3-chlorophenylmercapturic acid, 3,4-dichlorophenylmercapturic acid, benzoyl glucuronide and 2,2-dimethyl-2-phenethyl glucuronide); Dr C. J. W. Brooks (salicyluric acid).

Standards and reagents. Hexamethyldisilazane and trimethylchlorosilane were obtained from Applied Science Laboratories Inc., State College, Pa., U.S.A. Pyridine and acid anhydrides were the purest commercially available materials. Ethereal diazomethane was prepared from Diazald (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) in batches as required.

Straight-chain even-numbered hydrocarbons from C_8 to C_{32}, used as reference standards, were obtained from Aldrich Chemical Co. and Applied Science Laboratories Inc. Standardized reference mixtures of fatty acid methyl esters, prepared to National Institutes of Health specifications (Applied Science Laboratories Inc.), were used to evaluate the quantitative behaviour of the instrumental system.

The stationary phase, F-60 (Dow Corning 560 siloxane polymer) was obtained from Dow Corning Silicones, Midland, Mich., U.S.A.

Preparation of derivatives. All reactions involved in preparing derivatives for chromatography are carried out in tapered glass centrifuge tubes fitted with Teflon-lined screw caps and, unless otherwise stated, all reactions are carried out at room temperature. Because of the small volumes involved, all reactions in a series are carried out in the same tube without transfer, and if it is necessary to remove solvents or reagents this is done with the aid of a stream of dry nitrogen with the tube immersed in a water or air bath at about 40°. Because of the extreme water-sensitivity of some derivatives it is most important that the nitrogen be dry. The final trimethylsilylated reaction products are injected directly on to the column without removal of reagent. Excess of reagent is necessary to ensure quantitative reaction.

Methyl esters. About 1mg. of the acid (or about 1mg. each of a mixture of acids) is dissolved in 0·1 ml. of methanol. To the methanol solution is added a slight excess (usually 1–2ml.) of ethereal diazomethane, and the mixture is immediately evaporated to dryness under dry nitrogen. If simultaneous methylation of phenols is to be avoided it is essential that the diazomethane treatment be carried out as rapidly as possible. Exposure to diazomethane should always be less than 1 min. Methanol is a strong catalyst of diazomethane methylations and in our experience methyl ester formation is essentially an instantaneous reaction under these conditions.

Methyl ester formation with urine extracts is carried out in a similar way (Horning, Knox, Dalgliesh & Horning, 1966). The extract is dissolved in 0·5ml. of methanol and treated with ethereal diazomethane as described above.

Trimethylsilyl ethers. To the tube containing the residue after diazomethane treatment and removal of solvent is added 0·25ml. of pyridine, and to the resultant clear solution is added 0·15ml. of hexamethyldisilazane and 0·05ml. of trimethylchlorosilane. The tube is sealed, set aside at room temperature overnight, centrifuged, and the supernatant used directly for injection.

Urine extracts are treated similarly. However, it sometimes happens either that a clear solution is not obtained with pyridine, or that a cloudiness develops when hexamethyldisilazane is added. The amount of pyridine should then be increased sufficiently to give a clear solution after hexamethyldisilazane is added. (A cloudiness or precipitate, ammonium chloride, is always formed at the next stage, when trimethylchlorosilane is added. This does not affect the reaction either qualitatively or quantitatively.)

These are 'standard' conditions. In addition we use the following 'alternative' conditions. The acid (or mixture or urine extract) is taken up in 0·1ml. of dimethylformamide (or, if it does not dissolve, in the minimum amount needed to give a clear solution) and treated with ethereal diazomethane. The ether and excess of diazomethane are removed with dry nitrogen, and to the residue is added 0·15ml. of hexamethyldisilazane and 0·15ml. of pyridine (or such amounts of pyridine as may be necessary to give a clear solution; cf. above). The mixture is allowed to stand overnight before injection.

With urine extracts the 'alternative' procedure can result in larger volumes, which can be undesirable if sensitivity is paramount. However, if the substances being examined include aliphatic α-oxo acids the 'alternative' procedure is preferable. The 'standard' procedure can give rise to multiple derivatives with these substances.

The standard conditions give two reagent peaks with methylene unit values (see below) of 11·52 and 12·44 on a 10% F-60 column, and 11·62 and 12·50 on a 1% F-60 column.

Trimethylsilyl esters. The compound (or mixture or urine extract) is evaporated to dryness under dry nitrogen if in solution. To the dry residue is added 0·25ml. of pyridine, followed by hexamethyldisilazane and trimethylchlorosilane, as described for trimethylsilyl ethers. The same requirement for clear solutions applies. Hydroxyl groups are converted into trimethylsilyl ethers at the same time that carboxyl groups are converted into trimethylsilyl esters. Reaction mixtures should be left at least overnight to ensure complete reaction.

Propionylation. All carboxyl groups in the mixture or urine extract are converted into methyl ester groups as described above. The residue, after removal of solvent, is taken up in 0·35ml. of pyridine and 0·15ml. of propionic anhydride is added. The mixture is set aside overnight at room temperature, and injected without further treatment.

Preparation of urine extracts. Human urine samples (24hr.) were diluted to 2l. with deionized water; if the

volume was greater than 2l. no further dilution was made. Rat urine (8hr. collection) was diluted with distilled water to 20ml.

Portions of urine (10ml.) were added to 20ml. of saturated sodium chloride solution in a separating funnel, and the pH was adjusted to 1·0 with 6N-hydrochloric acid. The solution was extracted with three 25ml. portions of redistilled ethyl acetate followed by extraction with three 25ml. portions of ether. The combined ethyl acetate and ether extracts were dried with magnesium sulphate and filtered. The filtrate was evaporated to dryness under reduced pressure (Rotovap). The residue, containing extracted acids and neutral compounds, was transferred with redistilled methanol to a calibrated centrifuge tube fitted with a Teflon-lined screw cap. The volume of methanol was reduced to 0·5ml. with the aid of a nitrogen stream.

This procedure of Horning *et al.* (1966) extracts most aromatic acids in quantitative fashion. Appreciable amounts of hydrophilic substances such as citric acid and glucuronides are also extracted. Examples of the use of this method in analyses for the human and the rat are given in the study by Horning *et al.* (1966).

Preparation of columns. F-60 (Dow Corning 560 siloxane polymer) is coated on Gas Chrom P, 80–100 mesh (Applied Science Laboratories Inc.), as described in Horning, VandenHeuvel & Creech (1963).

To obtain the resolution necessary for quantitative assessment of complex mixtures attention to detail in column preparation is essential. In particular, the support should be accurately graded, acid-washed and silanized before application of the stationary phase. Glass columns should be silanized before packing, and the coated support should be packed evenly.

Glass U-tube columns 6ft. × 3·5mm. internal diam. are used. Packed columns are baked at 260° before use. A 1% column is usable after baking overnight, and a 10% column after baking for several days. However, 'bleed', especially at high temperatures, may be evident for a few days. Provided that it is regenerated when necessary (as described subsequently) a column should provide continuous service for several months. All columns showed satisfactory quantitation (within 1% of theoretical values) of a standard reference mixture of fatty acid methyl esters.

Ovens and electronic equipment. Columns are used in a Barber–Colman model 5000 one-column or two-column oven. Programming is carried out at a nominal 2°/min. (this rate of rise is found to be optimum for quantitation of substances emerging at widely differing temperatures). The initial temperature of the column is usually 100° (but is set at any value from 40° upwards for special purposes) and programming is continued to 250°, or lower if appropriate. Columns can be run up to 260° for brief periods, but this shortens their useful life.

The chromatographic effluent is monitored by a hydrogen flame-ionization detector with a Keithley electrometer (model 417) usually set to give full-scale deflexion at 3×10^{-10}A. The output of the electrometer is connected to a recorder with a chart speed 0·5in./min.

The temperature of the injection block is maintained at 250°, and the flame detector chamber at 300°. The pressures of the hydrogen and air supplies to the flame are usually 20 and 40lb./in.^{2} respectively. Nitrogen is the carrier gas and the pressure at the inlet is about 20lb./in.^{2}, the exact pressure appropriate to each column being determined by evaluation of its quantitative performance at different pressures around 20lb./in.^{2}. The flow rate of carrier gas through the column falls as the temperature rises, but this does not affect the ability to obtain quantitative analytical results (Horning, Maddock, Anthony & VandenHeuvel, 1963).

Mass spectrometry. Spectra are determined with an Atlas-Werke CH-4 mass spectrometer equipped with a gas-chromatographic inlet system developed by Ryhage (1964), which permits direct connexion of the gas chromatographic column and the mass spectrometer. When mass spectra are being determined, helium is used as carrier gas in place of nitrogen. The ion source is maintained at 250°. Ionizing potential and current are 20e.v. and 60μA respectively.

Methylene unit values. Even-numbered straight-chain hydrocarbons from C_8 to C_{32} are used as reference compounds. With F-60 columns programmed at 2°/min., provided that one is at least 25° above the temperature of injection, and therefore free of distortions due to the solvent front, the hydrocarbon peaks emerge at intervals which are close to linear (see Fig. 1). It is considered justifiable to assume linearity between two adjacent hydrocarbon standards. If the distance between the peaks of hydrocarbon C_n and C_{n+2} is x cm., and the distance from hydrocarbon peak C_n to the peak being measured is y cm., the methylene unit value is $n+(2y/x)$. Alternatively (and necessarily if conditions are used where the hydrocarbons do not emerge at approximately linear intervals, e.g. immediately adjacent to the solvent front) the methylene unit value can be determined graphically from a curve of the type of Fig. 1. Methylene unit values should be interpolated from hydrocarbon reference compounds differing by not more than two units. Methylene unit values can be converted into 'retention indices' (Kovats, 1958) by multiplying by 100. We consider methylene units preferable to Kovats indices and less confusing. Thus it seems to us simpler and clearer to say that the C_{22} hydrocarbon doco-

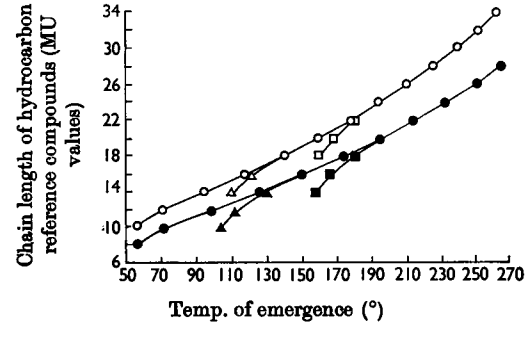


Fig. 1. Relationship between the temperature of emergence of *n*-alkane reference standards and methylene unit (MU) values (equivalent to chain length of the hydrocarbon). All values obtained on 6ft. columns of 10% F-60 (solid points) or 1% F-60 (open points) programmed at 2°/min. with initial temperature 50° (●, ○), 100° (▲, △) or 150° (■, □). Temperatures of emergence become independent of initial temperatures, above values which can be inferred from the points of convergence of the curves.

sane has a methylene unit value 22, rather than to say it has a Kovats retention index 2200.

RESULTS

Examples of the type of separation obtained by using the procedures described in this paper are given in Fig. 2, showing mixtures of known compounds, and Figs. 3 and 4, showing typical curves from urine extracts.

Choice of derivatives. The compounds of greatest interest in our metabolic investigations contained carboxylic acid groups (aromatic or aliphatic or both) or hydroxyl groups (phenolic, alcoholic, or potential enolic as in keto acids) or both. The choice of derivative was therefore largely governed by the requirement for quantitative reaction of these groups (for satisfactory gas chromatography, compounds with polar functional groups must be converted into volatile derivatives). Two alternative procedures were found satisfactory, both giving quantitative conversions of carboxyl or hydroxyl groups into single derivatives. These procedures were (*a*) conversion of carboxyl groups into methyl esters and subsequent conversion of hydroxyl and potential hydroxyl groups into

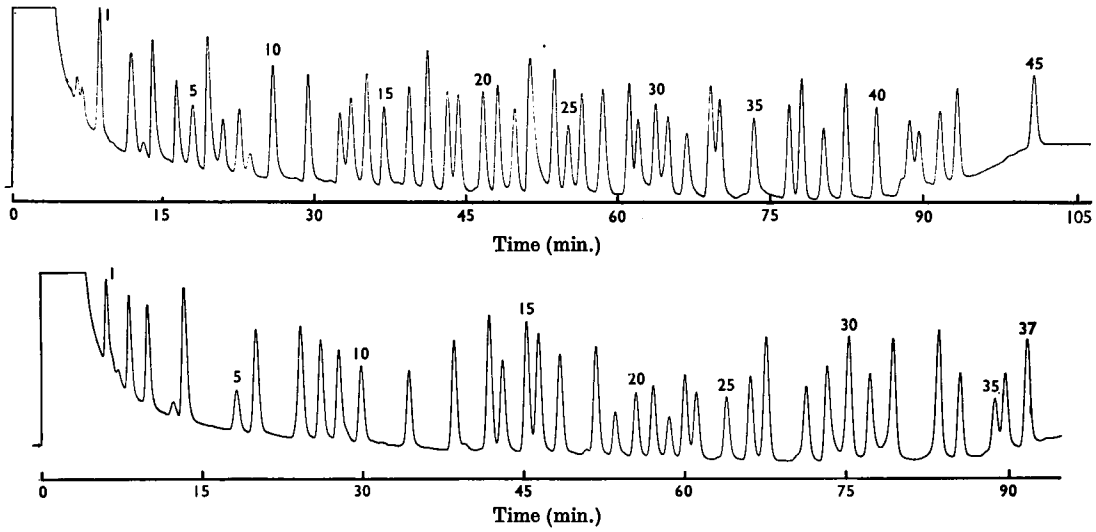


Fig. 2. Examples of separations of mixtures by the methods described in this paper. In the upper curve all carboxyl groups of mixture components are converted into methyl esters, whereas in the lower curve all carboxyl groups are converted into trimethylsilyl esters. In both curves all hydroxyl and phenol groups are converted into trimethylsilyl ethers. Both curves are run on 10% F-60, starting at 50° and programming at 2°/min. up to 250° or until the last peak emerges. It will be seen that under these conditions peak shape is remarkably consistent, and little affected either by chemical type or by temperature of emergence. The parent compounds represented on the curves are (reference numbers on the curves in parentheses): upper curve (methyl esters/trimethylsilyl ethers): decane (1): 2-hydroxyisovaleric acid; benzoic acid; octanoic acid; reagent peak A (see the text) (5); phenylacetic acid; dodecane; phenethyl alcohol; reagent peak B (see the text); malic acid (10); decanoic acid; cinnamic acid; β-hydroxy-β-methylglutaric acid; mandelic acid; *cis*-aconitic acid (15); tartaric acid; *m*-hydroxyphenylacetic acid; *p*-hydroxyphenylacetic acid; citric acid; isocitric acid (20); 3,4-dimethoxyphenylacetic acid; *p*-methoxymandelic acid; hippuric acid; 3,4-dihydroxybenzoic acid; 3,4-dihydroxyphenylacetic acid (25); *p*-hydroxycinnamic acid; D-quinic acid; vanilmandelic acid; indolylpropionic acid; 3,4-dihydroxymandelic acid (30); 3-methoxy-4-hydroxycinnamic (ferulic) acid; mannitol; 5-methoxyindolylacetic acid; indolyl-lactic acid; 5-hydroxyindolylacetic acid (35); docosane; nonadecanoic acid; indolylpyruvic acid; arachidic acid; tetracosane (40); androsterone; aetiocholanolone; dehydroepiandrosterone; hexacosane; octacosane (45). Lower curve (trimethylsilyl esters/trimethylsilyl ethers); isovaleric acid (1); valeric acid; tiglic acid; lactic acid; reagent peak A (see the text) (5); α-hydroxyisovaleric acid; benzoic acid superimposed on reagent peak B; octanoic acid; phenylacetic acid; succinic acid (10); tetradecane; decanoic acid; salicylic acid; cinnamic acid; *m*-hydroxybenzoic acid (15); phenyl-lactic acid; β-hydroxy-β-methylglutaric acid; tartaric acid; veratric acid; tridecanoic acid (20); *cis*-aconitic acid; *o*-hydroxycinnamic acid; 3,4-dihydroxybenzoic acid; citric acid; vanilmandelic acid (25); 3,4-dihydroxyphenylpropionic acid; ascorbic acid; *p*-hydroxyphenylpyruvic acid; 5-methoxyindolylacetic acid; 3,4-dihydroxycinnamic acid (30); 5-hydroxyindolylacetic acid; stearic acid; nonadecanoic acid; tetracosane; androsterone (35); aetiocholanolone; dehydroepiandrosterone (37).

trimethylsilyl ethers, and (*b*) conversion of carboxyl groups into trimethylsilyl esters and of hydroxyl and potential hydroxyl groups into trimethylsilyl ethers. A logical alternative way to cover hydroxyl groups would be by acylation. This, as discussed below, did not prove to be a satisfactory approach.

Other workers (Williams & Sweeley, 1964; Williams, 1965) have examined methyl esters/trimethylsilyl ethers as protecting groups for aromatic hydroxy acids, and have reported multiple derivatives and lack of quantitative results. It seems very probable that some of the multiple derivatives are methyl ethers (see section below on methylation). This problem can be reduced to an unimportant level if the procedure recommended here is used. Other apparent multiple derivatives are likely to be artifacts. For example, Williams (1965) made methyl ester/trimethylsilyl ether derivatives by treating first with hexamethyldisilazane and then with diazomethane. In our experience, apart from trimethylsilyl esters being formed, carrying out reactions in this order can give rise to whole series of peaks appearing at regular intervals, presumably arising from a polymerization reaction involving diazomethane. Moreover, the trimethylsilyl esters which are first formed can apparently undergo partial reaction with diazomethane.

Methylation. Methanol is a strong catalyst for the methylation of carboxylic acids by diazomethane, and methyl ester formation from diazomethane in the presence of methanol is very rapid. Unfortunately methyl ether formation from certain phenolic groups is also very rapid. This is exempli-

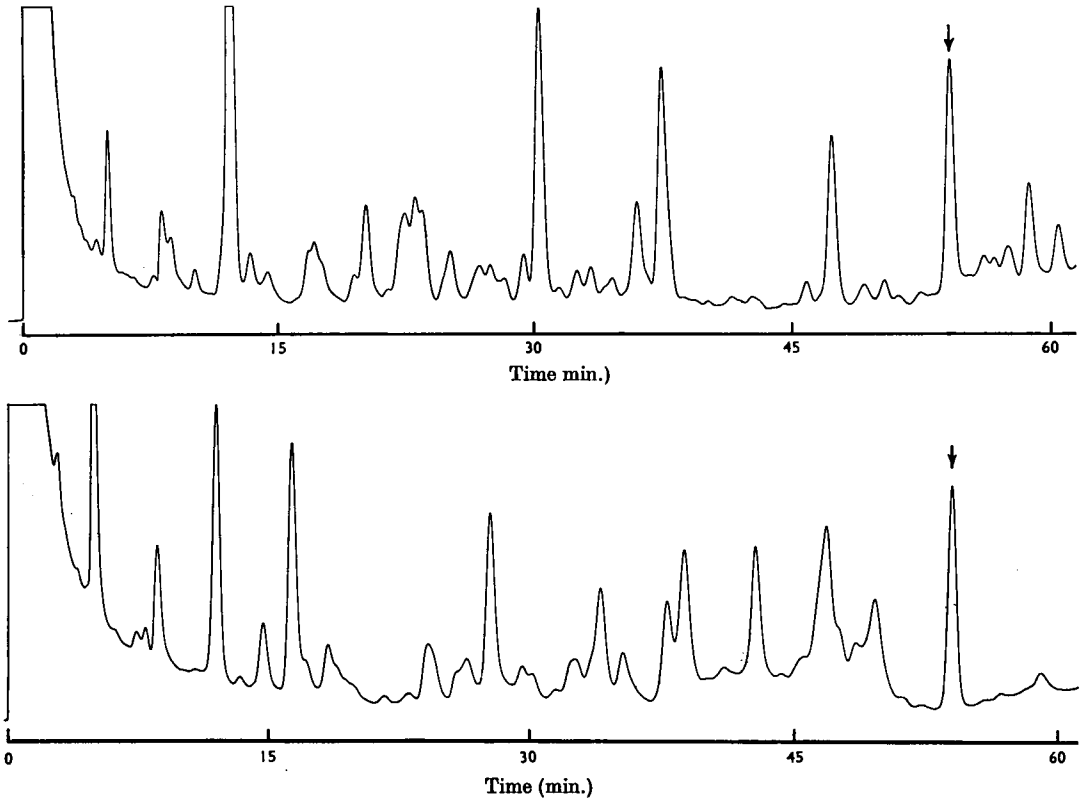


Fig. 3. Examples of the present technique applied to a human urine. A solvent extract (see the Experimental section) of the urine of a newborn male infant was divided into two parts. In one part (upper curve) all carboxyl groups were converted into methyl esters and all hydroxyl groups to trimethylsilyl ethers. In the other (lower curve) all carboxyl groups were converted into trimethylsilyl esters and all hydroxyl groups to trimethylsilyl ethers. To each extract eiocosane (indicated by the arrow) was added as an internal reference compound. Both chromatograms were run on 10% F-60, initial temperature 90°, with temperature programming at 2°/min. In each case 2 μl. of the derivative mixture was injected, corresponding to approx. 50 μl. of the original urine. The change in position of peaks on changing from methyl esters to trimethylsilyl esters facilitates identification of peaks and clarification of overlaps.

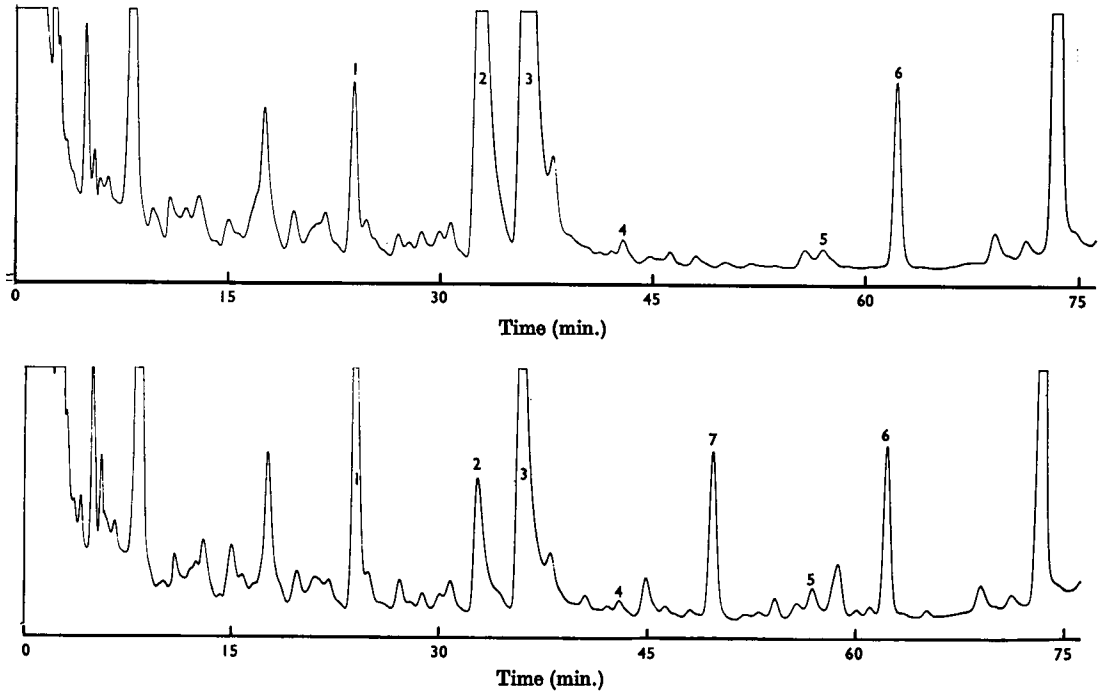


Fig. 4. Chromatograms of solvent extracts of 8 hr. urine collections from a 200 g. control rat (upper curve) and from a similar rat which had been given ethanol (6g./kg.) as an aq. 50% solution by stomach tube (lower curve). All carboxyl groups converted into methyl esters and all hydroxyl and phenolic groups into trimethylsilyl ethers. Nonadecanoic acid added as reference compound. Chromatograms run on 10% F-60, starting at 90° and programming at 2°/min. Injection volume 7 μl., corresponding to approx. 1/100 of the 8 hr. urine volume. Numbered peaks: *p*-hydroxyphenylacetic acid (1); hippuric acid (2); phenylaceturic acid (3); vanilmandelic acid (4); 5-hydroxyindolylacetic acid (5); nonadecanoic acid (6). After ethanol, there is a large rise in *p*-hydroxyphenylacetic acid (presumably derived from tyramine); a large fall in hippuric acid; a fall in phenylaceturic acid; in addition a new peak (7) appears, identified by its mass spectrum (for which we thank Dr I. Jaakonmaki) as ethanol glucuronide (ethyl β-D-glucosiduronic acid).

fied in Fig. 5. It will be seen that after 5 min. 3,4-dihydroxybenzoic acid has been largely converted into methyl ethers, and after 15 min. dimethylation is well advanced. The rate of methyl ether formation varies considerably with structure; for example, in the 3,4-dihydroxy series the rates of reaction are in the descending order 3,4-dihydroxybenzoic, 3,4-dihydroxyphenylacetic and 3,4-dihydroxymandelic. At first it was hoped to take advantage of the methylation of phenols to form derivatives for chromatography. But with a few phenolic groups the reaction is not quantitative even after long periods, and this approach was therefore abandoned.

We also examined other alcohols as catalysts. 2-Methylpropan-2-ol, for example, is a very much milder catalyst of esterification, and if methanol is replaced by 2-methylpropan-2-ol in the experiment illustrated in Fig. 5, even after 15 min. only a comparatively small amount of phenol ether formation has occurred. But though applicable to many artificial mixtures, 2-methylpropan-2-ol is not suitable for use with urine or tissue extracts because of solubility difficulties. A wide range of solvents were therefore examined for suitability in this reaction. The only solvents found which approached the solubility power of methanol for urine extracts were dimethylformamide and dimethyl sulphoxide.

In both reagents methylation of the phenolic group was slowed down. Dimethylformamide is the better solvent for the present purposes, but if the mixture requires to be concentrated it is difficult to remove under the mild conditions of time and temperature we regard as mandatory for urine and tissue extracts. If sensitivity of detection, and hence reaction volume, is important, we consider that the least objectionable procedure is to use

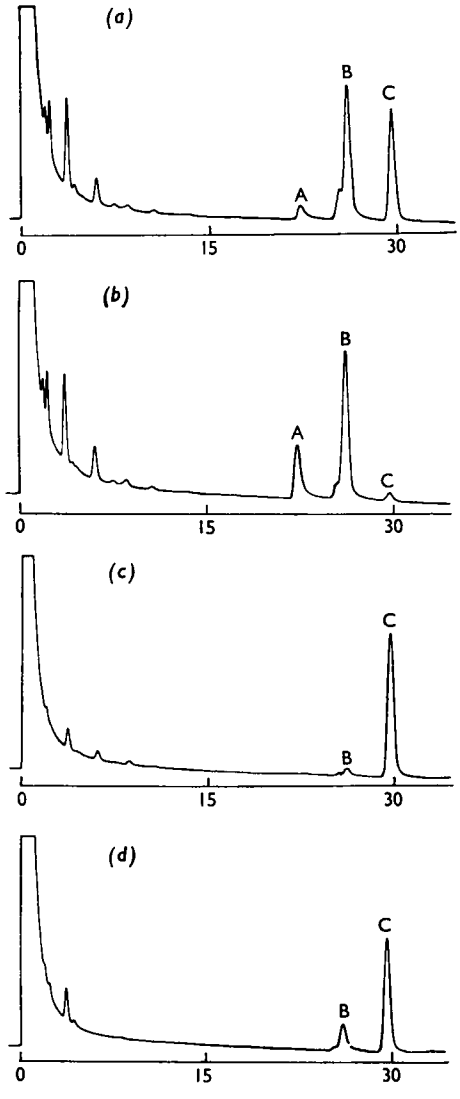


Fig. 5. Methylation of phenolic groups of 3,4-dihydroxybenzoic acid by ethereal diazomethane. All curves were run on 10% F-60 and programmed at 2°/min.: carboxyl groups converted into methyl esters and unmethylated phenolic groups into trimethylsilyl ethers. Peaks A, B and C represent 3,4-dimethoxy-(A), a mixture of the two isomeric methoxyhydroxy-(B), and 3,4-dihydroxy-(C) benzoic acids respectively. In the upper three curves (*a*–*c*) the solvent is methanol and in curve (*d*) is dimethylformamide. The dihydroxybenzoic acid solution was exposed to ethereal diazomethane: for 5 min. (*a* and *d*); for 15 min. (*b*); for the short period (*c*) recommended in the Experimental section ('standard' procedure). The high rate of methyl ether formation in methanol solution (cf. *a*, *b* and *c*) and the lower rate in dimethylformamide solution (cf. *d* and *a*) are evident.

methanol as solvent, but to work very rapidly as described in the Methods section. 3,4-Dihydroxybenzoic acid has the most rapidly methylated phenolic group that we have encountered and the low degree of methylation when the recommended 'standard' procedure is used is shown in Fig. 5. As 3,4-dihydroxy compounds likely to be of significance in urine have longer acid side chains and are methylated more slowly (see above) we think that methyl ether formation from significant metabolites can be kept to a level not interfering with quantitation.

If sensitivity, and hence reaction volume, is not a dominant consideration, undesirable methyl ether formation can be suppressed by using dimethylformamide as solvent in both methyl ester formation and trimethylsilyl ether formation, without removal of the dimethylformamide between reactions (the 'alternative' conditions; see the Methods section).

Trimethylsilyl esters and quantitative aspects of their formation. The reagent mixture used for converting hydroxyl groups into trimethylsilyl ethers will also convert carboxyl groups into trimethylsilyl esters. Such esters have already been used by Horii, Makita & Tamura (1965) for separating tricarboxylic acid-cycle intermediates by gas chromatography. The identity of these derivatives as esters is immediately established by the mass spectra. But the quantitative nature of the reaction is more difficult to assess.

Ideally, the response of a flame detector, as measured by peak area, should be proportional to the mass of the derivative passing through the flame. In fact, the flame does not 'see' all chemical groupings equally well, and this applies particularly to trimethylsilyl derivatives. Therefore when one is dealing with a variety of chemical types, as with urine and tissue extracts, it is necessary to determine a 'response factor' relative to some standard substance or substances, for each compound to be estimated. As standards we favour either *n*-alkanes or fatty acid methyl esters.

The extent of formation of derivatives can, of course, be demonstrated very simply when both unchanged and changed compounds show as peaks on the chromatogram. This is exemplified in the methylation experiments described above. The same method of examining the extent of reaction can usually be applied to compounds with 'difficult' (i.e. slowly reacting) hydroxyl groups; e.g. one can readily follow the trimethylsilylation of the tertiary hydroxyl group of citric acid methyl ester or the secondary hydroxyl group of mandelic acid methyl ester.

Demonstrating quantitative reaction of acid groups to trimethylsilyl esters is more difficult, as free acids do not give satisfactory peaks. That the products are trimethylsilyl esters is unambiguously

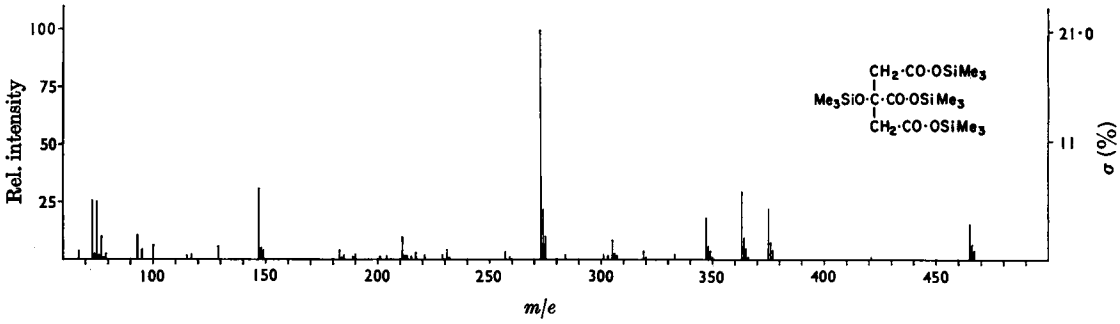


Fig. 6. The mass spectrum of citric acid trimethylsilyl ether tris-trimethylsilyl ester. The intensity values from the mass spectrum were fed (by using a Fortran programme) into an IBM model 1410 computer (International Business Machines, White Plains, N.Y., U.S.A.) driving an IBM model 1627 plotter. The computer calculated relative intensities, and plotted the curve (time required, approx. 40sec.). The compound shows no molecular ion at *m/e*480, but its correct molecular weight is indicated by the peaks at *m/e*465 (M-15) and *m/e*375 (M-15-90). This loss of 15 (due to Me) and 90 (due to Me_3SiOH) is commonly observed in the spectra of trimethylsilyl derivatives (e.g. Sharkey, Friedel & Langer, 1957; Eneroth, Hellström & Ryhage, 1964). Multiple peaks arising from the presence of the three silicon isotopes, ^{28}Si, ^{29}Si and ^{30}Si, are apparent.

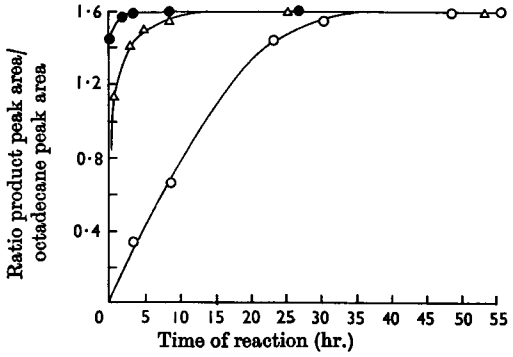


Fig. 7. Rate of formation of citric acid trimethylsilyl ether tris-trimethylsilyl ester under different conditions. Octadecane (in arbitrary molecular ratio) included as reference standard. ○, With hexamethyldisilazane (HMDS) alone at 65°; △, with HMDS and pyridine at room temperature; ●, with HMDS, pyridine and trimethylchlorosilane at room temperature. The first point on the last-mentioned curve corresponds to 5min., at which time the reaction is already 90% complete. The graph also provides evidence for the stability (at least up to 55hr.) of the reaction product in the presence of excess of reagent.

established by mass spectrometry. But as response factors cannot be predicted theoretically, one cannot deduce the extent of the reaction from the size of the peak. We have approached this problem kinetically, and we take citric acid as an example.

Citric acid treated with a suitable silylating mixture gives a sharp peak at 18·70 methylene units (on 10% F-60). The mass spectrum of this peak (Fig. 6) is consistent only with citric acid trimethylsilyl ether tris-trimethylsilyl ester. Formation of trimethylsilyl esters and ethers is brought about, though slowly, with hexamethyldisilazane alone. The reaction is much faster if pyridine is added to the reaction mixture (Makita & Wells, 1963; Sweeley, Bentley, Makita & Wells, 1963). It is much faster still if trimethylchlorosilane is also added as in the 'standard' procedure used in this paper. A quantitative (but arbitrary) mixture of citric acid and octadecane was therefore treated with these three different reagent mixtures and the appearance of citric acid trimethylsilyl ether tris-trimethylsilyl ester was followed kinetically by comparing the ratio of the peak area to the peak area of the octadecane. The results (Fig. 7) show that the end product from each reaction (which from the mass-spectrometric evidence is in every case the mono-ether tris-ester) is finally formed to the same extent, though the reaction rates are quite different. This we regard as good circumstantial evidence that quantitative reaction is finally achieved in each case. More definite evidence could be obtained if it were possible to isolate the end product chemically and show that the apparent response factor, assuming complete reaction, is equal to the true response factor. Unfortunately this is not practicable. Trimethylsilyl esters are stable (in our experience for quite long periods) in the presence of excess of reagent; but if the excess of reagent is removed the slightest trace of atmospheric moisture brings about their decomposition. We do not know the rate-limiting step in the overall reaction of citric acid. It has been claimed that elimination of water can occur on trimethylsilylation of tertiary hydroxyl groups (Friedman &

Kaufman, 1966). We have never observed this reaction. Thus, elimination of water from citric acid would give *cis*- or *trans*-aconitic acid, both of which give excellent peaks which would be readily detectable if they occurred.

An advantage of trimethylsilyl esters over methyl esters is that the problem of methyl ether formation from reactive phenols does not arise.

Acylation of hydroxyl groups. We have tried a wide range of acyl groups (straight- and branched-chain aliphatic, fluorinated and chlorinated aliphatic, aromatic) as hydroxyl-protecting groups. We have considered it essential that derivative formation should be carried out at room temperature to reduce the chance of destruction or modification of sensitive components of urine extracts (e.g. azlactone formation from glycine conjugates). With this restriction, all acylations tried have failed to a greater or lesser extent to react quantitatively with 'difficult' hydroxyl groups, e.g. the tertiary hydroxyl group of citric acid. It is, however, useful for qualitative purposes to have acyl compounds in reserve as alternative derivatives to solve particular separation problems on a qualitative basis, such as the clarification of overlaps. For room temperature acylation we prefer propionylation, and the chromatographic behaviour of some representative compounds is given in Table 5.

Characterization of peak position. The temperature of emergence of a peak, or the time for emergence when programmed at a given rate, are not satisfactory criteria for peak characterization. If two columns are compared in the same apparatus, or if the same column is used in different sets of apparatus, these values will be affected by such factors as slight differences in the amount of phase in two columns, slight differences in flow through columns due to differences in column packing, and differences in rate of programming (for example three ostensibly identical programmers set for 2°/min. give observed mean rates of rise of temperature of 1·76, 2·06 and 2·04°/min.). Two apparently 'identical' assemblies may therefore show differences in temperature of emergence of the same peak as great as 10° (though they are usually within 5°). However, a highly constant and reproducible parameter for peak characterization can be obtained by establishing the position of each peak relative to straight-chain hydrocarbon standards falling on either side. Thus a peak located six-tenths of the way between C_{18} and C_{19}, say, would be assigned a 'methylene unit' value 18·6. In

Table 1. *Methylene units of derivatives of carboxylic compounds*

Columns (A) and (B) give values for derivatives in which all carboxylic acid groups are converted into methyl (Me) ester groups. Columns (C) and (D) give values for trimethylsilyl (TMSi) esters. In all compounds any phenolic, alcoholic or potential enolic groups are converted into trimethylsilyl (TMSi) ethers. Compounds are arranged in the order of emergence of Me esters/TMSi ethers on 10% F-60, or of TMSi esters/TMSi ethers for compounds too small to be chromatographed as Me esters. MU, Methylene units.

	MU for Me esters/TMSi ethers		MU for TMSi esters/TMSi ethers		Increase of MU on changing from Me ester to TMSi ester
Parent acid	On 10% F-60 (A)	On 1% F-60 (B)	On 10% F-60 (C)	On 1% F-60 (D)	on 10% F-60 (C)−(A)
Butyric	*	*	8·52	*	
3-Methylbutyric	*	*	9·39	*	
Valeric	*	*	9·87	*	
2-Methyl-*trans*-butenoic (tiglic)	*	*	10·18	*	
Lactic	9·18	*	10·70	10·88	1·52
Hexanoic (caproic)	9·45	*	10·82	10·82	1·37
α-Hydroxyisobutyric (acetonic)	9·60	*	10·78	10·72	1·18
β-Hydroxybutyric	10·26	10·24	11·74	11·78	1·48
Oxalic	*	*	11·54	11·70	
Succinic	10·46	10·74	13·22	13·16	2·76
α-Oxobutyric	10·58	10·42	¶	¶	
α-Hydroxyisovaleric	10·58	10·50	11·80	11·92	1·22
Benzoic	10·92	10·94	12·42	12·44	1·50
Acetoacetic§	11·26	11·24	†	†	
α-Oxoisovaleric§	11·36	11·30	¶	¶	
Octanoic (caprylic)	11·38	11·54	12·74	12·84	1·36
Glutaric	11·38	11·50	14·20	14·28	2·82
α-Hydroxyisocaproic	11·48	11·52	12·58	12·72	1·10
α-Oxoisocaproic§	11·60	11·60	¶	¶	
Phenylacetic	11·74	11·84	13·00	12·98	1·26

Table 1 *continued*

Parent acid	MU for Me esters/TMSi ethers		MU for TMSi esters/TMSi ethers		Increase of MU on changing from Me ester to TMSi ester
	On 10% F-60 (A)	On 1% F-60 (B)	On 10% F-60 (C)	On 1% F-60 (D)	on 10% F-60 (C)−(A)
Oxaloacetic§	12·16	12·24	¶	¶	
Adipic	12·42	12·44	15·20	15·22	2·78
D(−)-Malic	12·72	12·88	15·26	15·34	2·54
Phenylpropionic	12·72	12·78	14·14	14·16	1·42
Decanoic (capric)	13·30	13·48	14·66	14·76	1·36
m-Methoxybenzoic	13·34	13·44	†	†	
Pimelic	13·42	13·44	16·20	16·30	2·78
p-Methoxybenzoic (anisic)	13·64	13·70	†	†	
Cinnamic	13·76	13·80	15·38	15·30	1·62
o-Methoxyphenylacetic	13·92	14·00	†	†	
β-Hydroxy-β-methylglutaric	13·94	14·12	16·30	16·32	2·36
Salicylic	14·00	14·08	15·14	15·04	1·14
Mandelic	14·10	14·20	14·86	14·80	0·76
m-Methoxyphenylacetic	14·16	14·28	15·20	15·16	1·04
α-Oxoglutaric§	14·24	14·28	16·36¶	16·54¶	2·12
trans-Aconitic	14·36	14·44	17·80	18·00	3·44
cis-Aconitic	14·36	14·50	17·82	18·00	3·46
p-Methoxyphenylacetic	14·38	14·44	†	†	
Suberic	14·48	14·54	17·16	17·28	2·68
m-Hydroxybenzoic	14·48	14·50	15·72	15·72	1·24
βδ-Dihydroxy-β-methylvaleric (mevalonic)‖	14·66	14·68	15·86	16·00	1·20
o-Hydroxyphenylacetic	14·74	14·74	15·74	15·72	1·00
(+)-Tartaric	14·74	14·82	16·84	17·00	2·10
Phenyl-lactic	14·80	14·78	16·00	16·00	1·20
p-Hydroxybenzoic	14·90	14·94	16·34	16·34	1·44
m-Hydroxyphenylacetic	15·08	15·18	16·18	16·24	1·10
p-Methoxyphenylpropionic	15·22	15·22	†	†	
Dodecanoic (lauric)	15·26	15·24	16·60	16·60	1·34
Dihydroxymaleic	15·26	15·20	17·20	17·30	1·94
p-Hydroxyphenylacetic	15·42	15·58	16·48	16·50	1·06
Azelaic	15·48	15·58	18·14	18·28	2·66
Citric	15·60	15·66	18·70	18·76	3·10
Fumaric	15·72‡	16·58‡	13·62	13·62	‡
3,4-Dimethoxybenzoic (veratric)	15·86	16·00	17·18	17·30	1·32
Indolylacetic	16·00	16·12	16·70	16·62	0·70
Isocitric	16·00	16·12	18·80	18·92	2·80
Phenylpyruvic§	16·00	16·00	17·16	17·12	1·16
Tridecanoic	16·26	16·32	17·58	17·58	1·32
3,4-Dimethoxyphenylacetic	16·30	16·44	17·26	17·40	0·96
p-Hydroxyphenylpropionic (phloretic)	16·36	16·42	17·66	17·62	1·30
3-Methoxy-4-hydroxybenzoic (vanillic)	16·50	16·52	17·72	17·72	1·22
2,6-Dihydroxybenzoic (γ-resorcylic)	16·50	16·44	18·00	17·88	1·50
p-Methoxymandelic	16·58	16·62	17·24	17·32	0·66
2,3-Dihydroxybenzoic (*o*-pyrocatechuic)	16·68	16·60	17·58	17·58	0·90
o-Hydroxycinnamic	16·70	16·82	18·14	18·18	1·44
3-Methoxy-4-hydroxyphenyl-acetic (homovanillic)	16·90	17·04	17·82	17·90	0·92
2,5-Dihydroxybenzoic (gentisic)	17·16	17·22	18·00	18·08	0·84
Tetradecanoic (myristic)	17·26	17·38	18·56	18·60	1.30
3,4-Dimethoxyphenylpropionic	17·30	17·26	†	†	
3,4-Dihydroxybenzoic (protocatechuic)	17·30	17·40	18·44	18·50	1·14

Table 1 *continued*

Parent acid	MU for Me esters/TMSi ethers		MU for TMSi esters/TMSi ethers		Increase of MU on changing from Me ester to TMSi ester
	On 10% F-60 (A)	On 1% F-60 (B)	On 10% F-60 (C)	On 1% F-60 (D)	on 10% F-60 (C)−(A)
2,4-Dihydroxybenzoic (β-resorcylic)	17·28	17·44	18·38	18·42	1·10
3,5-Dihydroxybenzoic (α-resorcylic)	17·48	17·56	18·48	18·62	1·00
p-Hydroxymandelic	17·52	17·66	18·08	18·26	0·56
3,4-Dihydroxyphenylacetic	17·56	17·60	18·48	18·44	0·92
2,5-Dihydroxyphenylacetic (homogentisic)‖	17·74	17·80	18·66	18·70	0·92
3-Methoxy-4-hydroxyphenyl-propionic	17·80	17·86	†	†	
p-Hydroxycinnamic	17·82	17·88	19·40	19·40	1·58
Shikimic	18·00	18·20	18·58	18·80	0·58
3,4-Dimethoxymandelic	18·18	18·38	†	†	
D-Quinic	18·22	18·40	19·10	19·28	0·88
Pentadecanoic	18·26	18·38	19·60	19·64	1·34
3,4-Dihydroxyphenyl propionic	18·48	18·52	19·62	19·66	1·14
3-Methoxy-4-hydroxymandelic (vanilmandelic)	18·64	18·76	19·16	19·36	0·52
3,4-Dimethoxycinnamic	18·70	18·90	20·28	20·40	1·58
Indolylpropionic	18·84	19·00	20·00	20·00	1·16
3,4-Dihydroxymandelic	19·16	19·30	19·70	19·86	0·54
p-Hydroxyphenylpyruvic§	19·16	19·34	20·74	20·82	1·58
Hexadecanoic (palmitic)	19·28	19·40	20·56	20·60	1·28
3-Methoxy-4-hydroxycinnamic (ferulic)	19·44	19·60	20·98	21·02	1·54
Indolylbutyric	19·86	19·90	21·26	21·18	1·40
3,4-Dihydroxycinnamic	20·12	20·18	21·56	21·64	1·44
Heptadecanoic	20·24	20·38	21·52	21·54	1·28
5-Methoxyindolylacetic	20·30	20·48	21·12	21·20	0·82
Indolyl-lactic	20·48	20·48	21·34	21·32	0·86
3,5-Dimethoxy-4-hydroxy-cinnamic (sinapic)	21·08	21·12	22·50	22·56	1·42
5-Hydroxyindolylacetic	21·18	21·30	22·00	22·00	0·82
Octadecanoic (stearic)	21·28	21·38	22·54	22·58	1·26
Nonadecanoic	22·26	22·34	23·56	23·54	1·30
Indolylacrylic** (I)	21·44	—	—	—	—
(II)	22·36	22·22	23·76	23·72	1·40
Indolylpyruvic§** (I)	22·80	22·82	—	—	—
(II)	23·70	23·58	24·64	24·52	0·94
Eicosanoic (arachidic)	23·26	23·30	24·50	24·54	1·24
Docasanoic (behenic)	25·26	25·28	26·46	26·52	1·20
Tetracosanoic (lignoceric)	27·21	27·28	†	†	

* The peak emerges at too low or too high a temperature for the particular derivative to be used on the particular column.

† Authentic material not available (usually applies to methyl ethers prepared by diazomethane methylation of phenols; simultaneous methyl ester formation occurred, and trimethylsilyl esters were therefore not made in these cases).

‡ Treatment of fumaric acid with diazomethane results in pyrazole formation as well as ester formation. Columns (A) and (B) therefore refer to a different type of derivative from columns (C) and (D).

§Keto acids form trimethylsilyl ethers of their enol forms. For Me esters/TMSi ethers of aliphatic keto acids the 'alternative' procedure of derivative preparation should be used; the 'standard' procedure gives multiple peaks. See text.

‖ The corresponding lactone is listed in Table 2.

¶ Not a satisfactory derivative, owing to formation of multiple peaks. See text.

** Compounds (I) and (II) are respectively without and with a trimethylsilyl group on the indole nitrogen atom.

Table 2. *Methylene units of derivatives of non-carboxylic compounds*

All phenolic, alcoholic and potential enolic OH groups are converted into trimethylsilyl (TMSi) ethers. MU, Methylene units.

Parent compound	MU of TMSi ether: On 10% F-60 (A)	On 1% F-60 (B)
Phenol	10·48	10·40
p-Cresol	11·46	11·42
Phenethyl alcohol	12·22	12·22
Glycerol	13·16	13·06
Mevalonolactone	13·62	13·62
Homogentisic lactone	17·46	17·66
Tryptophol	18·00	18·12
3,4-Dimethoxyphenylglycol	18·24	18·38
Methyl ascorbate*	18·54	18·62
3-Methoxy-4-hydroxy-phenylglycol	18·68	18·80
3,4-Dihydroxyphenylglycol	19·18	19·36
Mannitol	19·90	20·00
Ascorbic acid	19·92	20·00
Sorbitol	20·00	20·12
Dulcitol (galactitol)	20·00	20·18
5-Methoxytryptophol	20·32	20·32
5-Hydroxytryptophol	21·24	21·20
*myo*Inositol	21·42	21·46
Androsterone	24·76	24·44
Aetiocholanolone	25·06	24·76
Dehydroepiandrosterone	25·54	25·14
Cholesterol	†	30·80
Desmosterol	†	31·24

* Product obtained on treating ascorbic acid in methanol with ethereal diazomethane for a prolonged period. With the 'standard' conditions of rapid methylation a trace of this product may be formed.

† Peak emerges at too high a temperature for this derivative to be used with this particular column.

practice, because of their more ready availability, only even-numbered hydrocarbons are used as standards. The consistency of such values is impressive. In this Laboratory methylene units of a given compound determined by several different workers with different pieces of equipment using many different columns over a period of many months have only rarely been found to differ by as much as 0·04 and are usually within 0·02 (e.g. see Horning *et al.* 1966). The methylene unit value of a peak is considered to be of considerable diagnostic value in peak identification.

Methylene units are therefore used in this paper for characterization of peak position.

In Tables 1–5 are listed the values (methylene units) for compounds run under the alternative conditions of our standard procedure. In general, compounds with methylene unit values differing by 0·2 or more will be resolved; those differing by 0·1–0·2 will appear as twin peaks or shoulders, those differing by less than 0·1 will not be resolved. However, resolution tends to be somewhat better at lower than at higher temperatures. The values can be converted into approximate temperatures of emergence (with allowance for instrumental variation) by reference to Fig. 1.

Occasionally it is desired to run isothermal chromatograms to separate a limited number of similar compounds. A useful rule of thumb for determination of the appropriate temperature to be used is that it should be 3–5° below the temperature of emergence of the first relevant peak when programmed at 2°/min. This value, also, can be deduced from Fig. 1 and Tables 1–4.

Maintenance of column condition. Columns deteriorate, and it is therefore necessary to

Table 3. *Methylene units of derivatives of conjugates*

Columns (A) and (B) give values for derivatives in which all carboxylic acid groups are converted into methyl (Me) ester groups. Columns (C) and (D) give values for trimethylsilyl (TMSi) esters. In all compounds any phenolic, alcoholic or potential enolic groups are converted into trimethylsilyl (TMSi) ethers. Compounds are arranged in the order of emergence of Me esters/TMSi ethers on 10% F-60. Compounds containing amide groups show some trailing. MU, Methylene units.

Parent compound	MU for Me esters/TMSi ethers: On 10% F-60 (A)	On 1% F-60 (B)	MU for TMSi esters/TMSi ethers: On 10% F-60 (C)	On 1% F-60 (D)
N-Acetylglycine (aceturic acid)	11·76	12·42	13·16	13·64
N-Acetyl-L-glutamic acid	15·88	16·98	17·84	17·60
Hippuric acid (benzoylglycine)	16·92	17·50	18·40	18·60
o-Methoxyhippuric acid*	19·06	19·22		
Salicyluric acid* (*o*-hydroxyhippuric acid)	19·56	19·60	20·82	20·88
Anisuric acid (*p*-methoxyhippuric acid)	19·76	20·38	21·24	21·24
3-Chlorophenyl mercapturic acid	21·16	21·28	22·24	22·10
3,4-Dichlorophenyl mercapturic acid	22·64	22·66		23·42
Benzoyl glucuronide	25·20	25·32	25·46	25·58
2,2-Dimethyl-2-phenethyl glucuronide	26·22	26·26		

* For discussion of salicyluric derivatives see text.

Table 4. *Methylene units of derivatives of compounds containing pyridine rings, or amino substituents on benzene rings*

Columns (A) and (B) give values for derivatives in which all carboxylic acid groups are converted into methyl (Me) ester groups. Columns (C) and (D) give values for trimethylsilyl (TMSi) esters. In all compounds any phenolic, alcoholic or potential enolic groups are converted into trimethylsilyl (TMSi) ethers. Compounds are arranged in the order of emergence of Me esters/TMSi ethers on 10% F-60. Many of the peaks show some degree of trailing. MU, Methylene units.

	MU for Me esters/TMSi ethers		MU for TMSi esters/TMSi ethers	
Parent acid	On 10% F-60 (A)	On 1% F-60 (B)	On 10% F-60 (C)	On 1% F-60 (D)
Nicotinic	11·36	12·34	12·96	13·52
Picolinic	12·10	12·96	13·30	13·58
Anthranilic I	13·38	13·56	14·80	14·78
* II	15·12	15·04	16·28	16·28
p-Aminobenzoic I	14·92	15·66	16·40	16·54
* II	17·00	17·04	18·38	18·38
3-Hydroxyanthranilic*	16·32	16·24	17·62	17·58
Quinolinic	16·74	16·82	17·94	18·00

* The aminobenzoic acids give two peaks: peak I, a trailing peak, is the free Me ester; peak II, a symmetrical peak, is the *N*-TMSi derivative of the Me ester. 3-Hydroxyanthranilic gives only a single symmetrical peak.

Table 5. *Methylene unit values of derivatives in which any carboxylic acid groups are converted into methyl esters and any phenolic or alcoholic groups are propionylated*

Arranged in order of emergence from a 10% F-60 column programmed at 2°/min. MU, Methylene units.

	MU for derivative	
Parent compound	10% F-60 (A)	1% F-60 (B)
Salicylic acid	15·06	14·98
m-Hydroxybenzoic acid	15·72	15·68
Glycerol	16·06	16·18
Citric acid	16·48	16·50
p-Hydroxyphenylacetic acid	16·70	16·70
3-Methoxy-4-hydroxyphenylacetic (homovanillic)	18·36	18·78
2,5-Dihydroxybenzoic (gentisic) acid	19·64	19·62
Tryptophol	19·76	19·86
5-Methoxyindolylacetic acid	20·40	
3-Methoxy-4-hydroxymandelic (vanilmandelic) acid	21·38	21·50
5-Methoxytryptophol	22·00	
5-Hydroxyindolylacetic acid	23·18	
5-Hydroxytryptophol	24·98	25·72

re-evaluate their efficiencies at regular intervals. Deterioration may first become evident from loss of peak symmetry and increase in trailing. It is useful to have available a quantitative mixture containing both a substance such as 5-hydroxyindolylacetic acid, which is very sensitive to column condition, and a substance such as a fatty acid methyl ester, which is less sensitive. A change in ratio of peak area between these two can often give an indication of column deterioration before change in peak shape becomes obvious. Useful pairs in the trimethylsilyl ester series are citric acid (very sensitive) and a fatty acid (less sensitive).

If a column deteriorates, it is not necessary to discard it. The deleterious changes are usually confined to the upper 2–3in. of the column, and if this portion is removed and replaced by a fresh portion of the original packing the column is usually restored to its original efficiency. A short period of baking (2–4hr. for a 1% column or overnight for a 10% column) is necessary before re-use. However, deterioration of peak shape, or of peak size, may instead be due to minute leaks developing in stoppers, connexions or injection septa. A baking period is also required after changing any stoppers or connexions, to eliminate spurious peaks due, e.g., to plasticizers in stoppers.

Behaviour of specific substances and chemical types

Keto acids. Mass spectrometry establishes that methyl esters of all the keto acids in Table 1 are converted into trimethylsilyl ethers of their enol forms. In many cases this change can also readily be demonstrated chromatographically by comparing methyl esters before and after treatment with the silylating mixture.

With aliphatic α-oxo acids the 'standard' procedure can give multiple derivatives. This can be avoided by using the 'alternative' procedure (see

the Methods section) for all acids tried except pyruvic acid. When trimethylsilyl esters are prepared, all the aliphatic α-oxo acids can give multiple derivatives, whether 'standard' or 'alternative' conditions are used. Besides the expected trimethylsilyl ester trimethylsilyl enol ether, there are formed various nitrogen-containing derivatives, the structure of which is being investigated.

Fumaric acid. After treatment with diazomethane this gives a pyrazole derivative (as well as ester formation) by addition of CH_2N_2 to the double bond (McKeown & Read, 1965). The trimethylsilyl ester is a preferable derivative of this acid.

Pyrazole formation has not been observed with any of the other unsaturated acids in Table 1.

Glycine conjugates and mercapturic acids. All glycine conjugates tend to show slight trailing, this being more severe on 1% F-60 than on 10% F-60, and the degree of trailing is a good indication of column condition. Mercapturic acids trail rather less than hippuric acids. Numerous amide conjugates have been detected in urine, but in Table 3 are included only those for which authentic samples were available.

Salicyluric acid. The phenolic group is very readily methylated, and if diazomethane treatment is not carried out rapidly two peaks can be formed, of the methyl ester/methyl ether and the methyl ester/trimethylsilyl ether (the latter a symmetrical peak; structures confirmed by mass spectrometry). In contrast the phenolic group of unconjugated salicylic acid is rather slow to methylate.

Compounds slowly methylated. Certain non-phenolic non-carboxylic compounds can be methylated if exposure to diazomethane is over-prolonged, e.g. ascorbic acid.

Lactone formation. Certain *o*-phenolic acids, e.g. homogentisic acid, lactonize readily. Lactone formation is rarely observed if reactions are carried out with freshly prepared solutions.

Pyridine derivatives. These give trailing peaks resembling amides, even though they contain no free hydrogen atom on the nitrogen atom.

Aniline derivatives. Anthranilic acid and *p*-aminobenzoic acid give two derivatives, shown by mass spectrometry to be the free ester (a trailing peak), and the *N*-trimethylsilyl derivative (a symmetrical peak), respectively.

Indolylacrylic acid derivatives. Indolylacrylic acid gives two derivatives, shown by mass spectrometry to be with and without a *N*-trimethylsilyl group on the indole nitrogen atom. Indolylpyruvic acid (which in its enol ether form is also an indolylacrylic derivative) also gives a second peak if the reaction mixture stands for a long period. These are the only indoles which have shown indole *N*-trimethylsilylation under our conditions.

DISCUSSION

As we were seeking a method of wide general application it was necessary to select both derivatives and a stationary phase applicable to many chemical types. The application of gas chromatography to separative problems has been a major interest of this Institute (for reviews see Horning, VandenHeuvel & Creech, 1963; Horning, Luukkainen, Haahti, Creech & VandenHeuvel, 1963; VandenHeuvel & Horning, 1964, 1965; Horning & VandenHeuvel, 1964, 1966) and our exploratory investigations were greatly facilitated by availability of a large 'library' of different types of column, phase and equipment, and accumulated experience on the behaviour of different types of functional groups and derivatives on different phases. It soon became evident that polysiloxane phases were most suitable for dealing with the wide diversity of molecular types and sizes encountered in a urine extract, and of those available F-60 (Dow Corning 560) was found to be most effective. SE-30 is similar, but in our experience less satisfactory for the present purposes.

The wide range of molecular weights of metabolites in urine render single isothermal chromatograms impracticable, as any given temperature could only provide useful separation of a very small fraction of the metabolite range. This difficulty can be overcome by temperature programming. By using a rate of rise of temperature of 2°/min. with a 6ft. column, it has been found that peaks are very similar in shape over the entire range from 40° to 250°, and the resultant chromatograms can be used for quantitative estimation as well as for characterization of substances emerging at widely differing temperatures. The quantitative abilities of columns were evaluated by using standard mixtures of fatty acid methyl esters, and columns were only used if the observed values were within 1% of theoretical values.

The higher the sensitivity and specificity of a separative procedure applied to a complex mixture such as urine, the greater the number of components that are revealed, and any single chromatographic system is likely to give several overlaps. A general procedure must therefore provide alternative approaches which allow overlapping peaks to be resolved. There are two main approaches (*a*) changing the type of derivative or (*b*) changing the stationary phase. Changing columns in many instruments is inconvenient, and time can be lost waiting for a newly inserted column to equilibrate. Moreover an additional objective of this work was development of a procedure suitable for automated gas chromatography, for which successful equipment has been developed in this and other laboratories. Obviously the advantages of automation

would be lost if it were necessary to interrupt the cycle to change the column. We have therefore sought resolution of overlapping peaks by change of derivative rather than by change of column. To give greater flexibility to the procedure we have, however, determined the parameters of known compounds with both 10% F-60 and 1% F-60 columns. The more heavily loaded stationary phase of the 10% column is particularly suitable at the low-molecular-weight end of the scale.

The present procedure will allow separation (and quantitative estimation in all cases examined) of: aliphatic compounds such as fatty acids above butyric acid; di- and tri-carboxylic acids; hydroxy acids and keto acids; polyhydroxy and alicyclic compounds such as glycerol, inositol, quinic acid, shikimic acid, ascorbic acid and sugar alcohols; aromatic hydroxy and acidic compounds, both benzenoid and indolic; sesquiterpenes; steroids; glycine conjugates, mercapturic acids and glucuronides. The method does not provide satisfactory separation of iminazole derivatives, polypeptides or sulphate conjugates.

Structural information can be obtained by connecting the gas-chromatographic column to a mass spectrometer of appropriate design. The mass spectrum can give a great deal of structural information. For the work described in the present paper, in which known compounds were treated singly or in small groups with known reagents, the identity of a peak could be unequivocally established by simple determination of the molecular weight from the molecular ion, without the necessity for examining the detailed spectrum. The detailed spectra, and the molecular transitions involved, are, however, of considerable interest both in themselves, and as aids in the identification of unknown peaks in urine, and will be reported in separate papers from this Institute.

Figs. 2, 3 and 4 give an idea of the resolving power of the procedure. For chromatography, hydroxyl groups have been converted into trimethylsilyl ethers; in the upper curves of Figs. 2 and 3 and both curves of Fig. 4, carboxyl groups have been converted into methyl esters; in the lower curves of Figs. 2 and 3 carboxyl groups have been converted into trimethylsilyl esters. It will be seen from these Figures that the peaks are the same shape for quite different chemical types. This consistency of peak shape for different types of compound over the whole temperature range contributes greatly to ease of quantitative estimation. Some compounds, however, always show a trailing peak, in particular amides (see e.g. the peak for hippuric acid in Fig. 2). A trailing peak shown by any compound not having an 'active' hydrogen atom indicates column deterioration.

Inspection of the values in Tables 1–4 shows that correlations of methylene units and structure can readily be made, and this allows prediction of the chromatographic behaviour of related compounds. For example, changing from a mono-methyl ester to a mono-trimethylsilyl ester causes a change in methylene unit value of the order of 1 unit. This change is additive, and the changes for dicarboxylic acids are of the order of 2 units and for tricarboxylic acids of the order of 3 units. Comparison of the two types of esters can therefore give an indication of the number of carboxyl groups. This is shown in more detail in Table 6. Similarly if a phenol group is converted into its methyl ether the compound comes out about 0·8 methylene unit earlier (earlier because the unmethylated phenol is chromatographed as the heavier trimethylsilyl derivative), and this again is additive (e.g. see series such as veratric, vanillic, 3,4-dihydroxybenzoic, also illustrated in Fig. 5).

As a broad generalization F-60 separates compounds in the order of the molecular weights of the individual derivatives, and is only secondarily influenced by the chemical nature of functional groups. However, there is sufficient specificity to give useful separation of structural isomers. Fig. 8 shows the separation of monohydroxy and dihydroxybenzoic acids. *Ortho-*, *meta-* and *para*-hydroxybenzoic acids (and similarly *o-*, *m-* and *p*-isomers of homologues and analogues) are readily separated from each other. Five of the six dihydroxybenzoic acids can be separated, but 2,4- and 3,4-dihydroxybenzoic acids overlap. This disadvantage is not too serious as 2,4-dihydroxy compounds are not usually encountered in urine or tissue extracts.

Methylene unit values obtained with 1% and 10% columns show a close general resemblance. However, overlapping peaks on one often separate on the other. Amides, for example, show a relatively greater change in methylene unit value between 1% and 10% columns than do esters and ethers so that, for example, homovanillic acid and hippuric acid, which overlap on a 10% column, are readily separable on a 1% column. We prefer to achieve resolution of overlapping peaks by change of derivative, but such considerations can contribute to the initial choice of column.

The choice of column (1% or 10%) depends primarily on the type of compound of immediate interest. For low-molecular-weight compounds, e.g. succinic acid, fumaric acid or valeric acid, it is necessary to use a 10% column, and also to use the relatively high-molecular-weight trimethylsilyl esters as derivatives (methyl esters would appear in, or too close to, the solvent front). By using programming from, say 50° to 250°, a 10% column, trimethylsilyl esters of carboxyl groups, and trimethylsilyl ethers of hydroxy groups, one can

Table 6. *Use of changes in methylene unit values with change of ester derivative to indicate the number of carboxyl groups in the parent compound*

All methylene unit (MU) values are taken from columns (A) and (C) of Table 1, and refer to changes from methyl (Me) to trimethylsilyl (TMSi) ester, with a 10% column.

Category of acid		No. of examples	Increase in MU on changing from Me ester to TMSi ester: Range	Mean	Mean per carboxyl group
Monocarboxylic	(*a*) aliphatic and alicyclic	22	0·58–1·52	1·22	1·22
	(*b*) benzenoid	38	0·54–1·62	1·16	1·16
	(*c*) indolic	8	0·70–1·40	1·00	1·00
	(*d*) total, all types	68	0·54–1·62	1·16	1·16
Dicarboxylic (all aliphatic)		12	1·94–2·84	2·55	1·27
Tricarboxylic (all aliphatic)		4	2·80–3·46	3·20	1·06

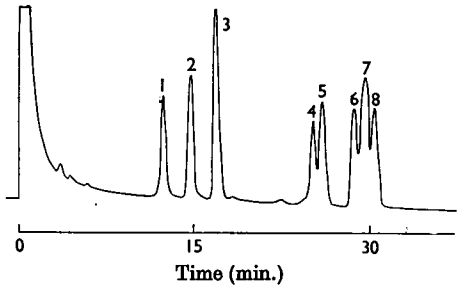


Fig. 8. Separation of *ortho-*, *meta-* and *para*-hydroxybenzoic acids (peaks 1, 2 and 3 respectively), and of dihydroxybenzoic acids: 2,6-dihydroxy (peak 4); 2,3-dihydroxy (peak 5); 2,5-dihydroxy (peak 6); mixture of 2,4- and 3,4-dihydroxy (peak 7); 3,5-dihydroxy (peak 8). Run on 10% F-60, programmed at 2°/min. All carboxyl groups were converted into methyl esters and all phenolic groups were converted into trimethylsilyl ethers.

cover the whole range from the C_4 acids up to compounds as large as 5-hydroxyindolylacetic acid, steroids such as androsterone, and the smaller aromatic conjugates (e.g. simple substituted hippuric acids, mercapturic acids or simple aryl or aroyl glucuronides). Higher-molecular-weight conjugates, or larger sterols such as cholesterol, would be outside the usable range, and to study these higher-molecular-weight compounds a 1% column is necessary. The useful ranges for different columns and starting temperatures can be deduced from Fig. 1. In considering the likely point of emergence one should consider the molecular weight of the derivative, rather than that of the parent compound. Mannitol, for example is chromatographed as its hexa-trimethylsilyl derivative, which has a molecular weight 614 compared with the molecular weight 182 for mannitol itself. The point of emergence is also markedly influenced by molecular shape, which in turn is influenced by aromaticity. The correlation between order of emergence, molecular weight, and aromaticity is illustrated in Fig. 9. In this Figure the methylene unit values on 10% F-60 are plotted against molecular weight of the derivative chromatographed for all compounds listed in Tables 1 and 2, except for esters of straight-chain mono- and di-carboxylic acids, and the hydrocarbons themselves. To avoid confusion these last-named compounds are correlated in Fig. 10. It is evident from Fig. 9 that there is a broad general correlation between molecular weight and methylene unit value but that the rate of increase of methylene unit value with increasing molecular weight is greater for aromatic than for non-aromatic compounds.

It will be seen that the aliphatic and alicyclic compounds all fall between the two lines marked A and A^1. The benzenoid compounds, with a few exceptions discussed below, all fall between the lines B and B^1. Indoles and steroids all fall between the lines C and C^1. In each group the spread for a given molecular weight is about 5 methylene units. On the other hand the straight-chain hydrocarbons and fatty acid esters (Fig. 10) show increases in methylene units with increasing molecular weight even steeper than the indoles. These results exemplify the effect of molecular shape on temperature of emergence. The aliphatic compounds falling between lines A and A^1, particularly the larger ones, tend to have multiple hydroxyl groups or carboxyl groups or both. These hydroxyl groups are converted into trimethylsilyl ethers for chromatography and the derivatives therefore have several bulky trimethylsilyl groups attached, which tend to make the molecule 'globular' in shape. On the other hand the benzenoid compounds will tend to be flatter and more rigid molecules with more 'spread' for a given molecular weight, and in the indole compounds this 'spread' will be even

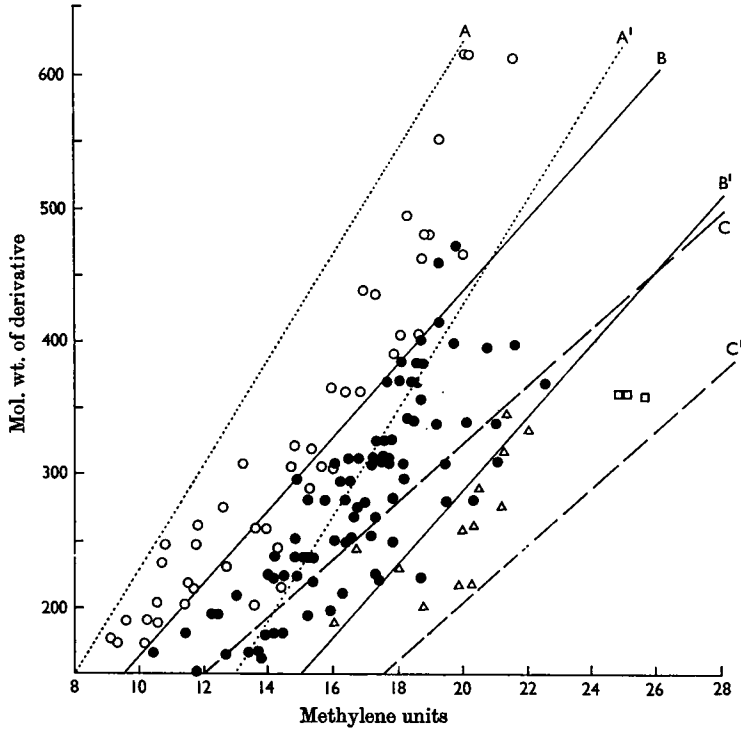


Fig 9. Correlation of methylene unit values on 10% F-60 with molecular weight of chromatographed derivative for substances in Tables 1 and 2 other than alkanoic and alkanedioic esters (for which see Fig. 10). ○, Aliphatic or alicyclic; ●, benzenoid; △, indolic; □, steroid.

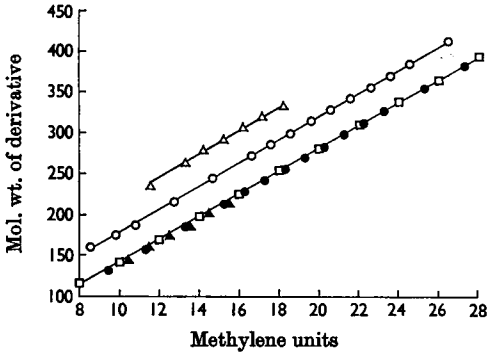
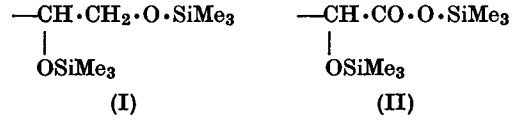


Fig. 10. Correlation of methylene unit values on 10% F-60 with molecular weight of chromatographed derivative: □, *n*-hydrocarbons (reference standards); ●, *n*-alkanoic acid methyl esters; ○, *n*-alkanoic acid trimethylsilyl esters; ▲, alkane-$\alpha\omega$-dioic acid methyl esters; △, alkane-$\alpha\omega$-dioic acid trimethylsilyl esters.

more marked. The exceptions amongst the benzenoid compounds are instructive. Those lying outside line B on the 'aliphatic' side are either phenylmandelic acid or phenylglycol derivatives which have immediately adjacent to the benzene ring one of the bulky groupings (I) and (II):



(I) (II)

This will obviously make the overall molecular shape tend towards the 'globular'. On the other hand, the exceptions on the 'indole' side of line B[1] are all cinnamic acids. Here the conjugation of the side-chain double bond with the aromatic ring increases the rigid planar area of the molecule and makes it comparable in size with the conjugated system of the indoles. Steroids behave as if they had an overall shape comparable with the indoles.

That the effect of aromaticity is primarily due to the effect that rigid aromatic systems have on molecular shape rather than due to aromaticity as such is suggested by Fig. 10. With long hydrocarbon chains the molecule can adopt an overall 'fibrous' shape, and with this type of molecule the increase in temperature of emergence with increas-

ing molecular weight appears to be maximal. It is interesting that hydrocarbons, fatty acid methyl esters and dicarboxylic acid methyl esters all lie very close to the same line. Presumably this is due to the fact that the cross-section of a CO_2Me group is approximately the same as the cross-section of a polymethylene chain.

Overlapping peaks can usually be resolved either by changing the column or changing the derivative. This is illustrated by considering two pairs of compounds which overlap when methyl esters/trimethylsilyl ethers are run on a 10% F-60 column; (*a*) homovanillic acid and hippuric acid and (*b*) 5-hydroxyindolylacetic acid and 5-hydroxytryptophol (these pairs represent the only overlaps disadvantageous for metabolic investigations which we have noted with this combination of derivative and column). Both pairs are resolved by changing from 10% F-60 to 1% F-60 (Tables 1, 2 and 3). For reasons stated above, we prefer not to change columns but instead to change derivative, e.g. from methyl esters to trimethylsilyl esters, which equally separates both pairs (Tables 1, 2 and 3). Or, if we are concerned only with identification and not with quantitative estimation, we can make the alternative change to methyl esters/propionyl derivatives (Table 5).

More difficult than the separation of overlapping peaks, however, is the problem of the detection of overlaps in the first place. This is best done by coupling the gas-chromatographic column to a mass spectrometer (Ryhage, 1964, and references therein). The time taken by the mass spectrometer to scan and record the range up to mass number 500 may be made as low as 1·5–2sec. Allowing time for the instrument to reset, a scan can readily be made every 3–4sec. Under the conditions of programming used in this paper the time from start to finish of emergence of a peak is approximately 1min. It is therefore possible to run numerous mass spectra on different parts of the same peak as it emerges. Inhomogeneity shows as changes in the identity of the mass peaks. When such inhomogeneity is evident the chromatogram can be repeated by using alternative derivatives.

Esters of straight-chain mono- and di-carboxylic acids, or *n*-hydrocarbons, are useful as internal standards for quantitative work. To be satisfactory, an internal standard should lie in an 'empty' part of the chromatogram. What part of a chromatogram will be 'empty' can be markedly altered by the nature of the pretreatment of the patient or animal, and availability of a wide range of possible alternative internal standards is therefore of value.

The method described in this paper was primarily developed for use in studies of mammalian metabolism, but it is likely to be equally applicable in plant or insect biochemistry. Thus substances such as quinic acid and shikimic acid, as well, of course, as phenols, give elegant peaks.

By comparing the behaviour of different derivatives of a complex mixture on a single column much can be learned from chromatographic evidence alone, and the use of several derivatives on the same column in an automatic machine running overnight is a considerable help in this connexion. Moreover, when the column can be connected with a mass spectrometer there results a highly sophisticated diagnostic tool. A substance present in microgram or submicrogram quantities in a complex mixture can be both estimated quantitatively by chromatography and structurally characterized by mass spectrometry. In those cases where it is not possible to deduce the detailed structure from the mass spectrum, one can still readily deduce such features as the accurate molecular weight, and whether or not the chromatographic peak is homogeneous. The deduction of structures from mass spectra will, of course, become much easier as the mass spectra of known metabolites in urine or tissue are established and interpreted, and this aspect is being pursued in this Institute.

The ability to separate, identify and measure, on the same chromatogram, such diverse substances as tricarboxylic acid-cycle intermediates, steroids and metabolites of biological amines (the same column, with different derivatives, can be used for the biological amines themselves; Capella & Horning, 1966) can be of great experimental value. These parameters can all vary in different ways in response to different types of stress. No single separative procedure can satisfy all needs. But the present procedure in our experience offers the possibility of examining metabolic patterns, and following the changes in these patterns in response to drugs, environmental change or other stimuli, on a broader scale than has previously been practicable.

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