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<i>Journal of Chromatography</i>	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	The publication schedule for further issues will be published later.						
<i>Chromatographic Reviews</i>			184/1	184/2									
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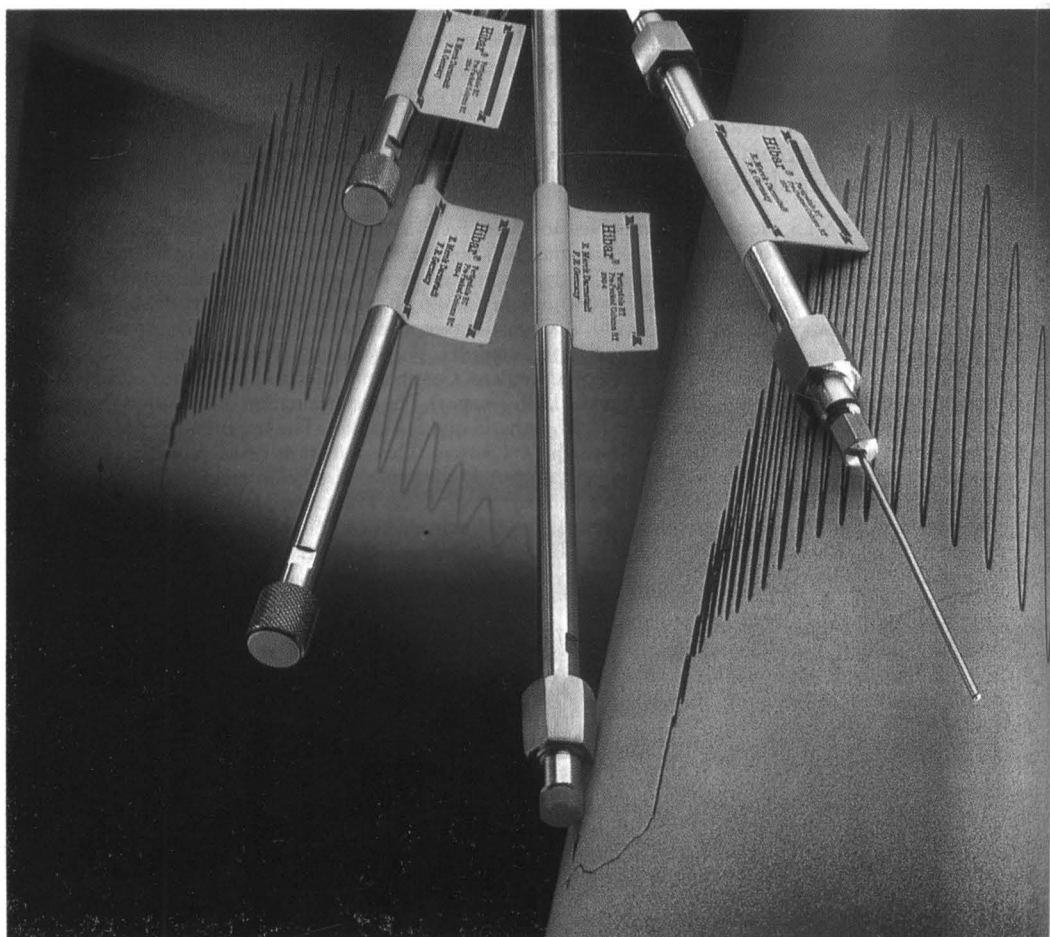
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CYCLIC DERIVATIVES FOR THE SELECTIVE CHROMATOGRAPHIC ANALYSIS OF BIFUNCTIONAL COMPOUNDS

COLIN F. POOLE and ALBERT ZLATKIS

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1. INTRODUCTION

This review is concerned with the chromatographic applications of chemically selective reagents forming cyclic derivatives with bifunctional compounds. The number of functionalized molecules in physiological fluids is immense and bifunctional compounds represent a small fraction of this physiologically important pool of substances. As such, they do not constitute a defined chemical class of substances like the steroids or terpenoids for example, but are found widely distributed among all classes of functionalized molecules (*e.g.* steroids, lipids, carbohydrates, nucleosides, catecholamines, prostaglandins, amino acids etc.). The distinctive physical feature that bifunctional compounds have in common is that they contain a minimum of two functional groups on a molecular framework which places these groups in close proximity to each other. No rigid geometrical rule can be given for the term "close proximity" as its meaning is related to the bridging ability of the reagent selected for the cyclization reaction. In general terms, bifunctional compounds can be considered to be those containing aliphatic chains with two functional groups on 1,2; 1,3; 1,4 carbon atom systems or aromatic compounds with *ortho* functional groups. The formation of larger rings than those represented by the above are rarely thermally or hydrolytically stable and are not suited for general chromatographic use.

As far as polar molecules are concerned, derivatization can be considered to be an auxillary technique in chromatography. Its impact on paper and thin-layer chromatography (TLC) has been small. Derivatives can be used to change relative mobilities of substances or to introduce a chromogenic tag to aid identification but neither technique is of necessity widely employed. In high-performance liquid chromatography (HPLC), most derivatization reactions are "detector orientated" and used to introduce UV-visible absorbing or fluorescent tags into substances with little or no native absorption in this region of the spectrum. This is a consequence of the current lack of a suitable universal and sensitive HPLC detector and the great reliance placed on the use of the UV-visible and fluorescence detector in routine analysis. It is probably in the gas chromatographic (GC) separation of polar molecules that derivatization has been most important. Initially derivatives were prepared to improve the thermal stability of polar molecules and to reduce their interaction with active centers in the chromatographic column and thus improve peak shape and separation characteristics. Derivatives have also been prepared to improve the resolution of complex mixtures by selectively removing a group of substances to a relatively empty region of the chromatogram and also as an aid to functional group identification through the characteristic changes in retention increments found after derivative formation with analysis on stationary phases of different polarity. Derivative formation is of considerable value in trace analysis as it provides a simple technique to introduce a tag into a molecule with a high response to a selective GC detector. This has considerably expanded the general usefulness and application of some detectors such as the electron-capture detector (ECD) into areas of research where it would otherwise have been of little value. The use of selective detectors and suitably designed derivatization reagents have a symbiotic relationship and both techniques have developed hand-in-glove with each other.

Early developments in derivatization were concerned with the search for universal one-step reagents which would react with all functional groups simultaneously.

The silylation and acylation reagents currently used today are successful examples of this research effort. Reagents of this type were designed to have the minimum of discriminatory power in chemical terms and consequently with complex mixtures they result in complex separation problems. In many analytical procedures, only a part of the sample is of interest and the rest (the matrix) is considered to be an interferent and must be separated either prior to analysis or by the resolving power of the chromatographic system. If the derivatization reaction could be made selective for the substance(s) of interest in the mixture and the reagent so designed that it could be used in conjunction with a selective detector for the determination, then the possibility exists for the development of simple analytical schemes for complex mixtures. Bifunctional compounds in mixtures could be selectively determined in this way.

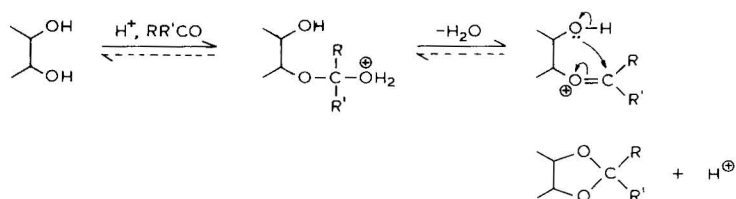
Information on selective reagents for the formation of cyclic derivatives has been recorded in general reviews of the techniques of derivatization¹⁻⁵. Darbre has reviewed the use of cyclization reactions in chromatography⁶.

2. ACETALS AND KETALS

Acetals and ketals are widely used in synthetic chemistry as protecting groups for *cis* diols and thiols. The derivatives are formed in high yield under mild conditions and are sufficiently stable to allow modification (*e.g.* esterification, oxidation, reduction etc.) to the underivatized portion of the molecule. The acetal and ketal protecting groups can be removed selectively by mild acid hydrolysis. The chemistry of the cyclic acetals of aldoses and aldoses^{7,8} and of teritols, pentitols and hexitols⁹ has been reviewed.

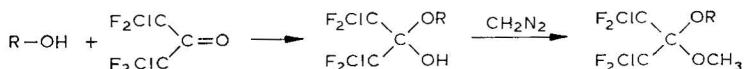
The impact of acetals and ketals in chromatography has been far less than their use in synthetic chemistry. Chromatography is often employed to monitor synthetic reactions involving acetals and ketals but this information is not specifically covered in this section unless the chromatographic step was important in the isolation or identification of the products.

Acetals and ketals are formed by the reaction between a ketone or aldehyde with a diol group on proximal carbon atoms. Generally, the ketone or aldehyde is used in excess, in many cases it may also be the solvent for the reaction, in the presence of an acid catalyst and/or a dehydrating reagent. Mechanistically, the reaction can be represented as follows:

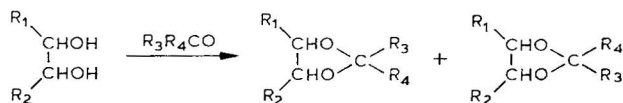


For aliphatic ketones and aldehydes, attack of the carbonium ion on the alcoholic oxygen atom results in the formation of a hemiketal which is rarely stable and rearranges to form the cyclic product. Substitution of the α -protons in the ketone or aldehyde with halogen atoms results in enhanced acidic character for the ketone group which confers much greater stability on the hemiketal intermediate and subse-

quent cyclization is no longer observed^{10,11}. The hemiacetal formed between *syn*-dichlorotetrafluoroacetone or hexafluoroacetone and steroid hydroxyl groups could be methylated with diazomethane and then used for the determination of the steroid by GC¹².



The condensation of an unsymmetrical glycol and either an aldehyde or an unsymmetrical ketone can result in the formation of two stereoisomeric forms which differ only in respect of their configuration at the new asymmetric carbon atom (originally the carbon atom of the carbonyl group).

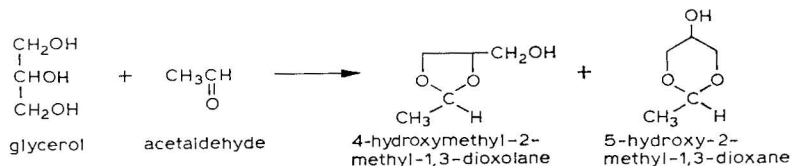


R₃, R₄ = H, alkyl or aryl group (R₃ = R₄ = H exempted)

The product from the reaction between a diol and acetone is variously called an acetonide or an O-isopropylidene derivative. For uniformity, the term acetonide is adopted throughout this review.

2.1. Derivatives of lipids, glycerides and hydroxyacids

The uropygiols (2,3-*n*-alkane diols containing 22–24 carbon atoms) of structure CH₃(CH₂)_{18–20}CHOHCHOHCH₃ were extracted from chicken green glands and identified in part by GC–mass spectrometry (MS) of the acetonide derivatives¹³. The three uropygiol acetonide derivatives were separated into six peaks on GC consisting approximately of equal amounts of the *threo* and *erythro* isomers. The prominent [M–15]⁺ ion in the mass spectra of the acetonide derivatives served to indicate the molecular weight of the original diol. The methyl ester of veronica oil (mainly *cis*-12, 13-epoxy-*cis*-O-octadecanoate) forms a diacetonide derivative separated into the two diastereoisomeric products on GC¹⁴. The double bond was epoxidized and then both epoxide groups converted to the acetonide with acetone and boron trifluoride–etherate catalyst. Wood¹⁵ has investigated the use of GC and TLC for the separation of unsaturated fatty acids by permanganate oxidation of the double bonds, methyl ester formation of the carboxylic acid group and acetonide formation of the double bond diols. The complex mixtures of *threo* and *erythro* isomers formed by fatty acids with multiple double bonds could only be partially resolved by GC and TLC. The acetonide derivatives of monounsaturated fatty acid esters were easily separated by GC. Positional acetonide derivatives, derived from positional isomers of monounsaturated fatty acids were not separated by GC but could be resolved by TLC.



Acetaldehyde and acetone react differently with glycerol¹⁶. Acetaldehyde

produces a mixture of four products corresponding to the *cis* and *trans* isomers of 4-hydroxymethyl-2-methyl-1,3-dioxolane and 5-hydroxy-2-methyl-1,3-dioxane which are separated by GC. Benzaldehyde has been shown to react in a similar manner with glycerol¹⁷. The reaction between glycerol and acetone produces only the dioxolane product. Acetonide derivatives have been prepared from α -monoglycerides (β -isomers do not react) esterified with C_2 – C_{18} fatty acids and separated by GC¹⁸. The acetonide derivatives of α -monoglycerides esterified with unsaturated fatty acids are eluted after their saturated homologues on the selective ethylene glycol succinate stationary phase¹⁹. These derivatives were stable to TLC and column chromatography using silicic acid. Ketal derivatives formed with acetone, methyl ethyl ketone, methyl isobutyl ketone, heptan-2-one, cyclopentanone and cyclohexanone have been used for the GC separation of mono and diepoxyglycerides²⁰. The epoxides were converted to the ketals by reaction of the appropriate ketone in the presence of boron trifluoride-etherate at room temperature for 2 h (*cf.* ref. 14). Under these conditions, conversion to the ketal was not complete; for example trivernolin gave 31 % of the 1,3-dioxolane derivative with cyclopentanone. For the analysis of natural products, cyclopentanone ketals were recommended as the epoxyglyceride derivatives were separated from the co-extracted C_{36} to C_{66} triglycerides.

The 2-hydroxy fatty acids from lipid hydrolyzates were determined by GC–MS after acetonide formation²¹. The 3-hydroxy fatty acids do not form acetonides and are easily differentiated from their 2-hydroxy analogues.

2.2. Derivatives of carbohydrates and nucleosides

Acetonide derivatives of fructose and glucose could be separated by paper chromatography²². The derivatives were formed using an ion-exchange catalyst but the reaction was unfortunately slow and did not always proceed to completion. In a more extensive study of the separation of carbohydrate acetonides by paper chromatography, good separations were obtained by reversed-phase ascending chromatography on cellulose acetate strips at 2–5° using methanol–water (6:4, v/v) as eluting solvent²³. Carbohydrates with different numbers of unreacted hydroxyl groups have characteristic mobility values (Table 1) which aids their identification. TLC has been used to monitor carbohydrate reactions and a few R_F values for carbohydrate acetonide derivatives given²⁴.

TABLE 1

REVERSED-PHASE PAPER CHROMATOGRAPHY OF SOME CARBOHYDRATE ACETONIDE DERIVATIVES

Derivative	R_F	Number of free hydroxyl groups
1,2:4,5-Diacetonide-3-mesyl-D-fructose	0.15	0
DL-Methylglycerate acetonide	0.28	0
1,2:3,4-Diacetonide-6-tosyl-D-galactose	0.32	0
Methyl-2,3-acetonide- α -D-ribofuranoside	0.48	1
1,2:5,6-Diacetonide-D-glucose	0.52	1
1,2:3,4-Diacetonide-D-galactose	0.55	1
1,2-Acetonide of glycerol	0.65	1
1,2-Acetonide-D-glucufuranose	0.80	3
3-O-Methyl-D-glucose	0.98	4

Hedgley *et al.*²⁵ were the first to use GC for the separation of carbohydrate acetonides. The authors described the separation on a preparative scale of the mono-acetonides of 5- and 6-deoxyglucose as very satisfactory and indicated that the method was applicable to the acetonide derivatives of hexoses and pentoses. Jones *et al.*²⁶ described the separation by GC of many acetonide, benzylidene, ethylidene and carbonate derivatives of carbohydrates. The derivatives were separated with free hydroxyl groups or after acetylation which reduced retention volumes. The separation of acetonide derivatives of carbohydrates has also been discussed by Kircher²⁷. Arzoumanian *et al.*²⁸ analyzed the 2,3-acetonide derivatives of methyl 5,6-dideoxy- β -D-allofuranoside-4-ene, methyl 5,6-dideoxy- β -D-glucofuranoside, methyl 6-deoxy- β -D-allofuranoside and methyl 6-deoxy- β -D-gulofuranoside by GC. Sweeley *et al.*²⁹ noted that acetals formed from anomeric sugar mixtures gave multiple peaks on GC and also that the benzylidene derivative of α and β -idosides was unsuitable for their resolution on a packed column of SE-52. It was shown that acetonide derivatives of carbohydrates with free hydroxyl groups may undergo rearrangement on GC unless the free hydroxyl groups are also protected by formation of acyl ester derivatives¹⁷. A brief report has appeared on the use of acetonide derivatives to determine monosaccharides in spent sulfite liquors³⁰. However, the method employed for the formation of the acetonide derivative was lengthy and unsuitable for routine use.

Butyraldehyde catalyzed by hydrobromic or hydrochloric acid reacts with derivatives of D-glucitol (1-dexoy-D-glucitol, 2-deoxy-D-glucitol and 3-O-methyl-D-glucitol) to give mixtures of products depending on the experimental conditions employed which could be separated by GC³¹. Benzylidene derivatives of monosaccharides have been prepared for the characterization of sugar *endo*- and *exo*-stereoisomers³². The benzylidene derivatives containing a free hydroxyl group gave a good separation of the isomers on GC; the *endo* isomers having shorter retention times.

Syrupy mixtures of aldoses (fucose, arabinose, xylose, rhamnose, galactose, glucose, mannose) could be converted to their acetonide derivatives at room temperature by a 2-h reaction with acetone containing 1% sulfuric acid as catalyst and the derivatives completely separated by GC (Table 2)³³. For glucose and rhamnose, a small peak, in addition to the main peak was observed and glucose also gave traces of a third product.

The retention times of 2',3'-acetonide derivatives of uridine and adenosine have been reported³⁴. The electron-impact mass spectrum of uridine, adenosine, cytidine and guanosine as their acetonides, acetonide trimethylsilyl ether, acetonide acetate and acetonide trifluoroacetate derivatives have been recorded³⁵. The acetonide group showed a characteristic elimination of a methyl group and acetone from the molecular ion.

The acetonide derivatives of carbohydrates are structural isomers as opposed to the stereoisomers formed with monodentate reagents and as such show strong characteristic and diagnostic modes of fragmentation in MS. Many mass spectra of the acetonide derivatives have been recorded and only the most salient features of these spectra will be dealt with here. For a more complete treatment, specialist reviews on the mass spectra of carbohydrates should be consulted^{8,36-38}.

The electron-impact mass spectra of carbohydrate acetonides have weak or absent molecular ions. A fragment ion at $[M-15]^+$ due to loss of a methyl group from the acetonide ring serves to identify the molecular weight of the original sugar.

TABLE 2

CHROMATOGRAPHIC DATA FOR GC OF ALDOSE ACETONIDE DERIVATIVES

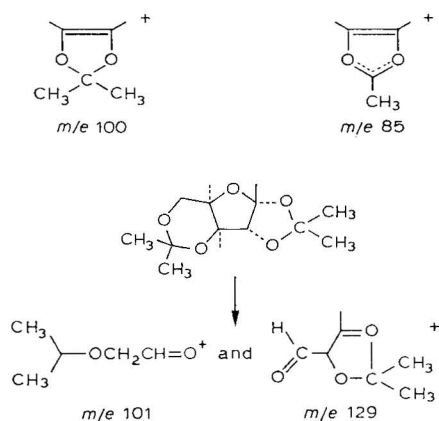
Aldose	Main acetonide	Relative retention time*	
		OV-225**	XE-60***
L-Fucose	1,2:3,4(α)	0.20	0.32
L-Arabinose	1,2:3,4(β)	0.24	0.38
D-Xylose	1,2:3,5(α)	0.39	0.49
L-Rhamnose	2,3	0.72	0.82, (0.78)
D-Galactose	1,2:3,4(α)	0.86	0.90
D-Glucose	1,2:5,6(α)	0.90 (0.82, 0.59)	0.94 (0.87, 0.60)
D-Mannose	2,3:5,6	1.00	1.00

* 2,3:5,6-Diacetonide of D-mannose used as internal standard.

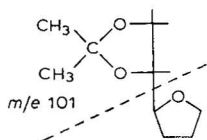
** 2 m \times 2 mm I.D. column of 3% OV-225 on Gas-Chrom Q (80–100 mesh), nitrogen flow-rate 20 ml min⁻¹. Temperature programme: isothermal, hold at 110° for 12 min, then 1.5° min⁻¹. Retention time of internal standard approximately 39.0 min.

*** 6 ft. \times 1.5 mm I.D. column of 3% XE-60 on Chromosorb G (80–100 mesh), nitrogen flow-rate 20 ml min⁻¹. Temperature programme: isothermal, hold at 120° for 5.0 min, then 2° min⁻¹. Retention time of internal standard approximately 34.0 min.

Further fragmentation of this ion by sequential elimination of the elements of acetone and acetic acid enable the number of acetonide groups in the molecule to be identified. Prominent ions of m/e 43 (C₂H₃O) and m/e 59 (C₃H₇O, protonated acetone) are characteristic of the acetonide ring. Diacetonide derivatives have characteristic ions at m/e 100 and m/e 85 while the monoacetonide derivatives usually only have the ion of m/e 85. The diacetonide derivatives of pentoses and hexoses undergo characteristic fragmentation ("h-rupture") to produce ions of m/e 101, 129 and 159.



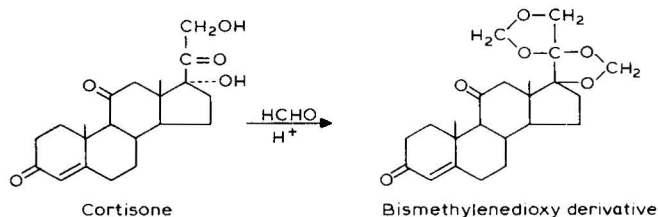
The acetonide derivatives can be used to distinguish between the furanose and pyranose isomers of sugars by mass spectrometry. The furanose acetonide derivatives



show a characteristic fragmentation involving rupture of the C-4, C-5 bond to produce a stable ion at m/e 101³⁹⁻⁴². This ion is only of diagnostic importance when relatively abundant.

2.3. Derivatives of steroids

The C-17 side chain of the corticosteroids is thermally labile and for the GC analysis of these compounds appropriate derivatives have to be prepared. Cortisone reacts slowly with formalin in acid media to form bismethylenedioxy derivatives⁴³.



As well as cortisone a series of 17-hydroxycorticosteroids were investigated and in nearly all cases one major peak was obtained on GC attended by shoulders or small secondary peaks. The results obtained with corticosteroids containing ring hydroxyl groups were unsatisfactory even after the formation of acetate derivatives. Corticosteroids with *cis*-C-20,21- or -C-17,20-dihydroxyl groups form acetonide derivatives under mild conditions⁴⁴⁻⁴⁶. The acetonide derivatives are suitable for separation by TLC and GC. The acetonide group was also stable to the conditions necessary to form trimethylsilyl (TMS) ethers, acetates or methoximes of other functional groups present in the molecule. The reaction was specific for *cis*-diols as the *trans*-C-20,21-diol did not form an acetonide derivative. Estetrol (1,3,5(10)-estratriene-3,15 α ,16 α ,17 β -tetrol) forms a 15 α ,16 α -acetonide derivative which is stable to aqueous acid and base and to the conditions necessary for the formation of the TMS ethers of the remaining hydroxyl groups^{47,48}. Trimethylchlorosilane was used as catalyst for acetonide formation. The epimers of estriols with *cis*-C-16,17-dihydroxyl groups are well separated as their acetonide derivatives on TLC using silica gel layers modified with ammonium bisulfite⁴⁹. The *trans* epimers do not form acetonide derivatives.

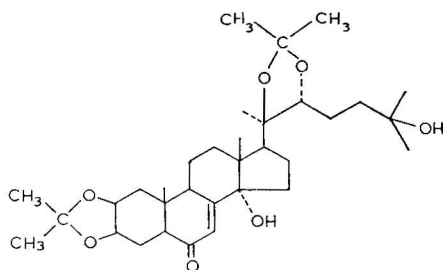
Mono- and diacetonide derivatives have been employed for the TLC separation and identification of the insect moulting hormones, ecdysones^{50,51}. The mass spectra of these derivatives have been recorded^{50,52}. Acetonide derivatives were used to confirm the presence of 3-epiecdysterone in the meconium fluid of the tobacco

TABLE 3

RELATIVE RETENTION TIMES OF 2 β ,3 β -DIHYDROXY-5 α -CHOLESTANE DERIVATIVES
3 ft. column of 1% OV-101 on Gas-Chrom Q, column temperature 260° and nitrogen flow-rate 80 ml min⁻¹.

Compound	Retention time (min)
2 β ,3 β -Dihydroxy-5 α -cholestane	2.40
Acetonide derivative	2.10
Di-TMS derivative	2.70
<i>n</i> -Butaneboronate derivative	3.60

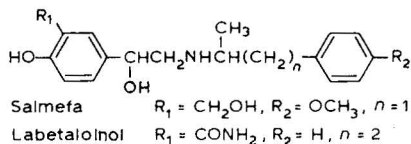
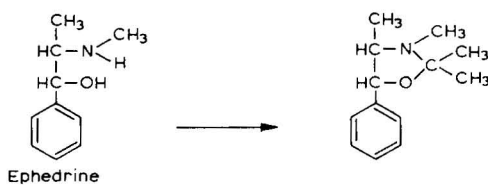
hornworm⁵³. The *trans*-2 β ,3 α -diol in this hormone does not form an acetonide derivative and this was used with other evidence to distinguish this hormone from ecdysterone. A convenient micro method has been developed for the formation of acetonide derivatives of ecdysone analogues⁵⁴. The steroid (0.2 mg) was dissolved in acetone (500 μ l) and phosphomolybdic acid added as catalyst (6 μ l of a solution containing 1 μ g/ μ l in acetone). The reaction was complete in 30 min at room temperature. The acetonide derivatives were more volatile than the TMS ethers or *n*-butaneboronates (Table 3). The acetonide derivatives were cleaved by trimethylsilylimidazole under mild conditions.



2,3:20,22-diacetonide derivative of ecdysterone

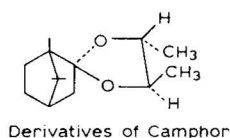
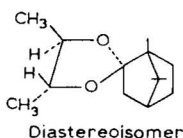
2.4. Derivatives of catecholamines and amino alcohols

Ephedrine and related catecholamines with at least one unsubstituted proton on nitrogen can be converted to acetonide derivatives which are stable to GC⁵⁵⁻⁵⁷. In one case, silica gel was used as catalyst and the reaction was complete in 6 h at reflux in acetone⁵⁷. The diastereoisomers of *l*-ephedrine and *d*-pseudoephedrine were reported as sharp peaks on a PEG 6000 column. Norephedrine reacts with acetone to form the Schiff base and methylephedrine does not react under the conditions employed for acetonide formation. Salmefanol and labetalol react with acetone, benzaldehyde and anisaldehyde to form cyclic derivatives which were found to be unstable towards GC⁵⁸.

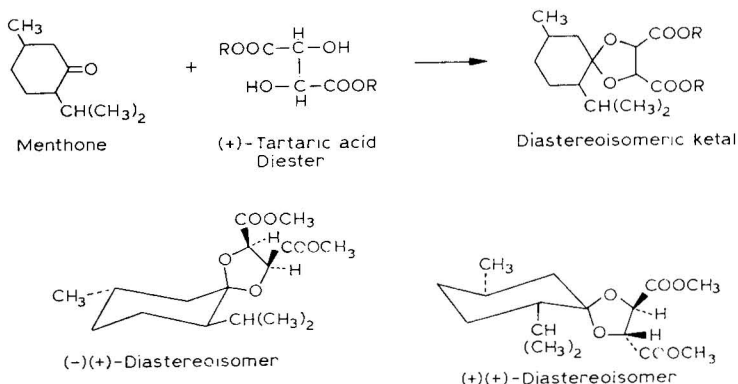


2.5. Derivatives for enantiomer separation

\pm Camphor reacts with D-(−)-2,3-butanediol in the presence of *p*-toluenesulfonic acid to give the two possible diastereoisomers which were separated by GC on both an analytical and preparative scale⁵⁹. Glass capillary columns were used to re-

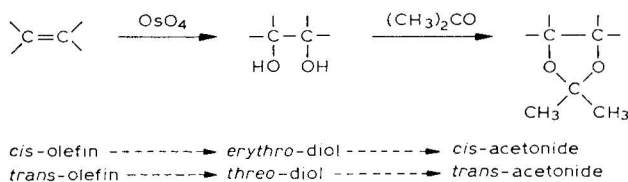


solve the diastereoisomeric ketals formed between (+)-dimethyl tartrate or (+)-2,3-butanediol and *sec.*-butylmethylketone, 2-methylcyclohexanone, 3-methylcyclohexanone, 2,4,4-trimethylcyclopentanone and 3,3,5-trimethylcyclohexanone⁶⁰. The diastereoisomers formed between (\pm)-menthone and (+)-tartaric diesters (dimethyl, diethyl, diisopropyl and dibutyl) were separated by GC using packed columns of Carbowax 20 M⁶¹. The resolution obtained with 2,3-butanediol was poor and the use of (+)-dimethyl tartrate was found to give the best separation. The (+)(+)-diastereoisomers always had longer retention times than the (-)(+)-diastereoisomers for the tartaric diester reagents used.



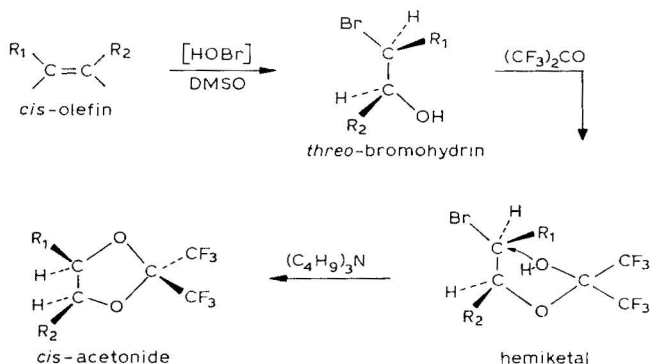
2.6. Derivatives for double bond location in alkenes

The location of the position of a double bond in a long-chain alkene by MS is difficult. Under the conditions prevailing in the mass spectrometer, the ionized molecular ion undergoes facile isomerization prior to fragmentation with a scrambling of the information concerning the location of the double bond and its geometrical configuration. The net result is a series of near identical mass spectra from which structural interpretation is impossible. A solution to this problem is the formation of derivatives of the double bond in a stereospecific way which yield fragments characteristic of the location of the double bond and intensity differences characteristic of the geometric arrangement of substituents attached to it.

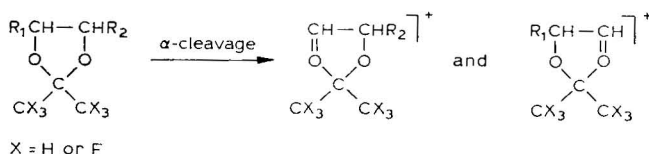


The double bond can be conveniently oxidized in olefins⁶² and unsaturated fatty esters^{63,54}, stereospecifically to the *cis*-diol, with osmium tetroxide. The diol can then

be converted to the acetonide derivative or cyclic boronate (see section on boronic acids) without changing the original configurational identity of the double bond. The use of Os^{18}O_4 and d^6 -acetone enables heavy isotope labeling to be conveniently carried out as an aid to mass spectral interpretation. Hexafluoroacetone does not condense with diols and acetonide derivatives with this reagent are formed via the bromohydrin¹¹. The bromohydrin adduct is *trans* specific (at least 97 %) and formation of the hexafluoroacetone ketal involves base-promoted backside attack on the bromine containing carbon atom with inversion of the configuration. A disadvantage of this reaction is the long time (2–3 days) required for derivatization and the moderate overall yield (85 %). The advantages are that the derivatives are volatile with good GC properties and the presence of fluorine enables fragmentation pathways in the mass spectra involving the acetonide ring to be easily distinguished from those occurring in the hydrocarbon portion.

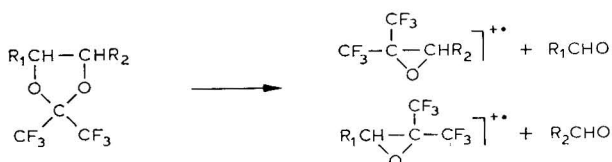


The mass spectra of all derivatives are characterized by a weak or absent molecular ion and a prominent $[\text{M}-\text{CH}_3]^+$ ion ($\text{M}-\text{CF}_3$ in the case of the hexafluoroacetone derivative) which serves to indicate the molecular weight of the original alkene. The tertiary carbonium ion formed is well stabilized by the two ether oxygen atoms and this constitutes a localized charged center from which the principal modes of fragmentation are initiated. Further loss of ketene (m/e 42) results in the formation of a protonated epoxide ion (not observed with hexafluoroacetone acetonides) and also loss of acetic acid in the case of the fatty acid esters (not observed with alkenes). In the lower mass range, two of the most prominent ions observed in all spectra m/e 43 (acetyl ion) and m/e 59 (protonated acetone) are formed from the acetonide ring (not always prominent as their fluorinated analogues in hexafluoroacetone acetonides).



The position of the double bond in the molecule is indicated by simple α -cleavage to form two ions containing either of the end groups of the original double bond. For the hexafluoroacetone derivatives the presence of ions 29 a.m.u. below the fragments formed by α -cleavage permits an unequivocal assignment of the double

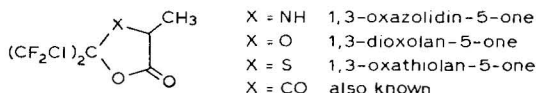
bond in the original alkene to be made. In the mass spectra of acetonides, secondary ions are formed from the fragments of α -cleavage as well as mass discrimination effects (substituents with the bulkiest R group tend to be more intense). For dienoid and trienoid unsaturated fatty acid acetonides, the number of double bonds can be established from the consecutive losses of acetone (m/e 58) from the $[M-15]^+$ ion. The position of the double bonds is less obvious as only those ions containing solely one acetonide ring are observed by α -cleavage⁶⁴. An interpretation can be made from the lower mass hydrocarbon fragments but this is less convenient and more prone to error.



As might be expected, the *erythro* and *threo* isomers of monoalkenes give rise to mass spectra which show intensity but not mass differences. However, unless suitable standards are available for comparison and the information is coupled with retention index values obtained by GC, the assignment of geometrical configuration is difficult^{11,63,64}. As a general observation, the acetonide formed from a *cis* double bond produces more prominent ions by α -cleavage than does the *trans* isomer^{11,62-64}. Intensity differences are usually in the range of two- to four-fold.

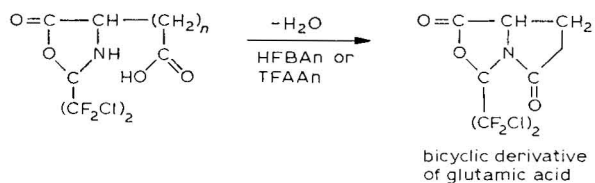
3. OXAZOLIDINONES

Substitution of hydrogen with electronegative groups such as chlorine or fluorine in acetone enhances the acidic character of the carbonyl group and promotes the formation of a series of stable monofunctional adducts and cyclic derivatives not observed with aliphatic ketones¹⁰. The condensation of hexafluoroacetone⁶⁵ and 1,3-dichlorotetrafluoroacetone^{65,66} with α -substituted carboxylic acids leads to the formation of a series of stable five-membered ring derivatives.

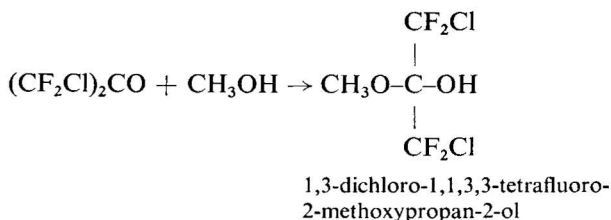


Some of these derivatives were found to be suitable for gas chromatography after silylation of protonic groups in the side chain with hexamethyldisilazane⁶⁵⁻⁶⁷. For the preparation of amino acid derivatives (X = NH), the conditions employed required elevated temperatures, long reaction times and polar solvents. The amino acid hydrochloride salts could not be derivatized under these conditions. Hušek investigated the reaction conditions for the formation of the oxazolidinones of tyrosine and its 3-iodo and 3,5-diiodo analogues^{68,79}. The solubilizing power of the solvent was found to be important and the addition of a trace of pyridine (1% in acetonitrile) rapidly dissolved the amino acid, its ammonium salt or hydrochloride salt enabling the condensation reaction with 1,3-dichlorotetrafluoroacetone to proceed smoothly and rapidly at room temperature. The use of strong bases such as triethylamine or N-methylpyrroli-

dine in place of pyridine resulted in a loss of derivative. Silylation of the phenolic group of tyrosine with N,O-bis(trimethylsilyl) acetamide gave unsatisfactory results. The preferred method was acylation of the phenolic group with trifluoroacetic anhydride or heptafluorobutyric anhydride which was carried out by addition of the anhydride to the condensation medium without removal of excess reagent. The derivatives so formed were very sensitive to the ECD and could be determined at the picogram level⁷⁰. With dicarboxylic amino acids such as aspartic acid, glutamic acid, aminoadipic acid and 2-aminopimelic acid, the use of heptafluorobutyric anhydride to derivatize the unreacted polar groups of the oxazolidinones gave unsatisfactory results⁷¹. No peak was observed for aspartic acid and multiple peaks were observed for the other dicarboxylic amino acids. This was explained by the possibility of the formation of bicyclic derivatives under the influence of the strong dehydrating action of the acid anhydride.



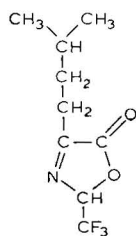
Derivatization of the second carboxylic acid group was carried out without significant by-product formation by addition of an alcohol to the condensation medium. For example, the addition of methanol immediately gave the expected hemiacetal, which becomes the reagent for the esterification reaction.



Further protonic groups in the side chain can be acylated with heptafluorobutyric anhydride after extraction and purification of the oxazolidine ester to give derivatives suitable for GC. As the condensation reaction occurs under mild conditions in weakly basic media, attack of the amino acid amide group was not observed making the method suitable for the simultaneous analysis of asparagine and glutamine in admixture with aspartic and glutamic acids⁷².

Amino acids (α -aminobutyric acid, α -amino- α -methylbutyric acid, alanine,

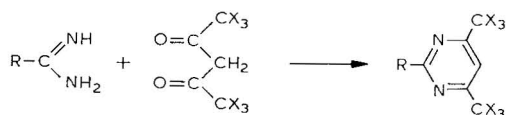
leucine, alloisoleucine, valine, cycloleucine, phenylglycine and phenylalanine) upon heating in a sealed tube with trifluoroacetic anhydride (150° for 10 min) have been shown to form inner esters with a 4-substituted-2-trifluoromethyloxazoline-5-one structure^{73,74}. The 2-trifluoromethyloxazolin-5-ones were more volatile than the N(O)-trifluoroacetyl-O-butyl ester derivatives and were well separated on GC. This reaction has been applied to the measurement of phenylalanine in serum⁷⁵. None of the amino acids investigated had protonic groups in the side chain.



Leucine oxazolinone derivative

4. CYCLIC DERIVATIVES OF BIGUANIDES

Biguanides of pharmaceutical importance can be converted to cyclic derivatives suitable for GC by condensation with acetylacetone⁷⁶⁻⁷⁹ or hexafluoroacetylacetone⁷⁹⁻⁸¹ to form substituted pyrimidines. The structures of the biguanides studied are summarized in Table 4.

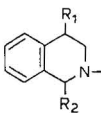
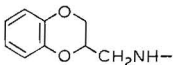
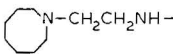
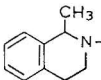
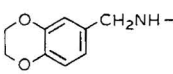


X = H or F

Acetylacetone can be made to react directly with biguanides in aqueous physiological fluids at slightly alkaline pH (sodium bicarbonate buffer)^{78,79}. The reaction is incomplete and for quantitative analysis a related substance is required as an internal standard^{77,79,82}. An alternative method (used with hexafluoroacetylacetone) employs two phases using benzene or toluene as the extraction solvent for the derivative. An advantage of this method is that very few interfering substances are extracted from blood or urine. For studies on the metabolism of biguanides in physiological fluids, very sensitive techniques are required and selective GC detectors are frequently used. Debrisoquine was determined with the nitrogen-phosphorus detector (NPD) in urine, plasma and saliva with a minimum measurable concentration of 3.0 ng ml^{-1} (ref. 79). Using mass fragmentography and tetradeuterio-debrisoquine as an internal standard, the least detectable amount of debrisoquine and its 4-hydroxy metabolite were 1.0 ng ml^{-1} and 5.0 ng ml^{-1} in plasma respectively^{78,81}. The detection limit of the hexafluoroacetylacetone derivative of guanabenzodioxan was 25 ng ml^{-1} with the ECD and approximately one order of magnitude lower when determined by mass fragmentography in biological fluids⁸². The minimum detectable concentration of debrisoquine hexafluoroacetylacetone derivative was $5 \cdot 10^{-12} \text{ g}$ of drug on column (corresponding to 25 ng ml^{-1} in plasma) with the ECD. Modification

TABLE 4

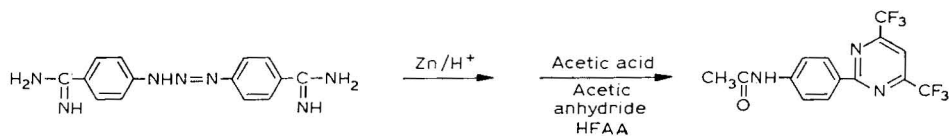
BIGUANIDES OF STRUCTURE $RC(NH)NH_2$ FORMING SUBSTITUTED PYRIMIDINE DERIVATIVES

<i>R Group</i>	<i>Name</i>	<i>Reagent</i>	<i>Ref.</i>
$-NH_2$	Guanidine	Acetylacetone	76, 83
$CH_3 NH-$	Methylguanidine	Acetylacetone	76, 83
$(CH_3)_2 N-$	Dimethylguanidine	Acetylacetone	76
	$R_1 = R_2 = H$, Debrisoquine $R_1 = OH, R_2 = H$, 4-Hydroxydebrisoquine	Acetylacetone	77 79
	Guanoxan Guanethidine	Acetylacetone Hexafluoroacetylacetone	79 80, 82
	3,4-Dihydro-1-methyl 2(1 <i>H</i>)-isoquinoline-carboxamide	Acetylacetone	79
	Guabenzoidioxan	Hexafluoroacetylacetone	82
			

of the extraction procedure should enable much lower concentrations in biological fluids to be determined⁸¹.

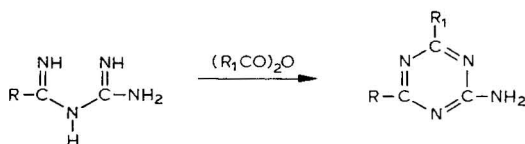
Seven derivatives (hexafluoroacetylacetone (HFAA), trifluoroacetylacetone, acetylacetone, trifluoroacetyl, acetyl, silyl, isothiocyanate) were evaluated for the GC analysis of guanidine, methylguanidine and agmatine⁸³. The cyclic derivatives had the most favorable properties of which the hexafluoroacetylacetone derivatives were recommended as these were the most volatile and hydrolytically stable. For derivatization a sealed tube reaction at 120° for 1 h with pyridine-HFAA (1:1) gave essentially complete reaction (100% for guanidine, methylguanidine and 97.4% for agmatine). With the AFID the least detectable amount of guanidine and methylguanidine was 5.0 ng and 15.0 ng for agmatine. With the ECD detection limits were 50 pg for guanidine, methylguanidine and 150 pg for agmatine.

Diminazene has been determined as the acetyl hexafluoropyrimidine derivative of 4-aminobenzamidine in plasma after a double acid reduction treatment in which the pyrimidine derivative is formed in the second stage in acid solution⁸⁴.



Diminazene could not be extracted from aqueous solution even at high pH and a double reduction technique in acid solution was necessary to ensure complete conversion to 4-aminobenzamidine otherwise unsatisfactory results were obtained. By chemical ionization MS using methane as the reagent gas and monitoring the ion $[MH]^+$, diminazene levels in the range 0.1 to 10 $\mu\text{g ml}^{-1}$ of plasma were determined.

The reaction between biguanides and organic acid anhydrides results in the formation of cyclic 2,4-disubstituted-2,6-amino-1,3,5-*s*-triazines which have good thermal and GC properties⁸⁵⁻⁸⁷. The structures of the biguanides studied are summarized in Table 5. The reaction of eight biguanides by either heating with anhydride or in a sealed tube reaction with anhydride and triethylamine to give yields of between 50–90 % of the *s*-triazine have been studied and the products characterized by elemental



analysis and physical spectroscopy (UV, NMR, MS)⁸⁶. The acid anhydrides tested include chlorofluoroacetic, dichlorofluoroacetic, heptafluorobutyric, pentafluoropropionic and trifluoroacetic anhydride. For the analysis of biological fluids, the drugs are first extracted from plasma after protein precipitation and basification into an organic solvent for derivative formation. Using chlorodifluoroacetic anhydride as cyclizing reagent, phenformin could be determined with an ECD at below the 1.0 ng ml^{-1} level in plasma⁸⁵. Using trifluoroacetic anhydride as cyclizing reagent and chemical ionization mass fragmentography 1–5.0 ng ml^{-1} of phenformin were determined in plasma⁸⁷. Under electron-impact conditions, most of the *s*-triazines fragment by α -cleavage to give the base peak of the mass spectrum and it has been suggested that this would be suitable for the analysis of physiological levels by mass fragmentography⁸⁶.

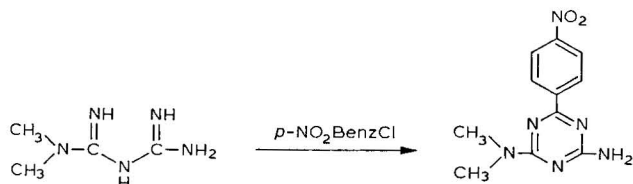
Metformin has also been determined by HPLC after formation of the UV-

TABLE 5

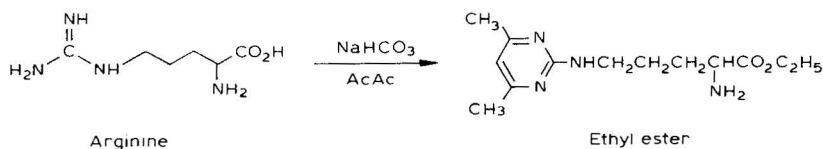
BIGUANIDES OF STRUCTURE $\text{RC}(\text{NH})\text{NHC}(\text{NH})\text{NH}_2$ FORMING CYCLIC SUBSTITUTED *s*-TRIAZINE DERIVATIVES

<i>R</i>	Name	Ref.
$\text{C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{NH}-$	Phenformin	85, 87
$\text{C}_4\text{H}_9\text{NH}-$	Buformin	85, 86
$(\text{CH}_3)_2\text{N}-$	Metformin	86, 88
$\text{C}_3\text{H}_7\text{NH}-$	1-Propylbiguanide	86
$\text{C}_6\text{H}_5\text{CH}_2\text{NH}-$	Benzylbiguanide	86
$\text{CH}_3\text{C}_6\text{H}_4\text{CH}_2\text{CH}_2\text{NH}-$	1-(<i>p</i> -Methylphenylethyl) biguanide	87
$\text{CH}_3\text{OC}_6\text{H}_4\text{CH}_2\text{CH}_2\text{NH}-$	1-(<i>p</i> -Methoxyphenylethyl) biguanide	87
$\text{C}_6\text{H}_5\text{CH}_2\text{CHNH}-$ CH_3	<i>d</i> -1-(α -Methylphenylethyl) biguanide	87
$\text{C}_5\text{H}_{11}\text{NH}-$	1-Pentylbiguanide	87
$\text{C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{C}_2\text{H}_5\text{N}-$	Amformin	87

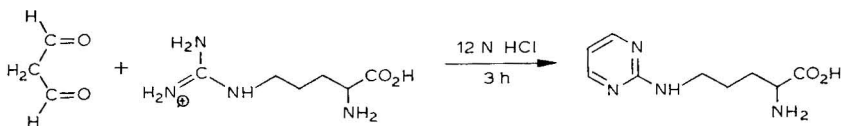
sensitive *p*-nitrobenzoyl derivative by addition of *p*-nitrobenzoyl chloride to urine made alkaline with sodium hydroxide⁸⁸.



Concentrations of methformin as low as 0.02 mg 100 ml⁻¹ of urine were determined in this way. Methylguanidine, guanidine and other guanidino compounds react with 9, 10-phenanthrenequinone to form a highly fluorescent product which can be detected at the low nanomole level (0.49 ng for guanidine, 0.63 ng for methylguanidine) with a fluorescence detector after separation of the guanidino compounds by cation-exchange HPLC^{89,90}. The derivatives were formed by a post-column reaction in a stainless steel delay coil (1 min at 75°). The method was suitable for determining guanido compounds in as little as 1.0 ml of serum or 0.2 ml of cerebrospinal fluid obtained from uremic patients.



The guanidino function of the amino acid arginine reacts with acetylacetone to form a pyrimidine derivative which after esterification of the carboxylic acid function is suitable for the analysis of arginine by paper chromatography and GC⁹¹. MS can be used to determine the sequence of all common amino acids in polypeptides except for arginine which exhibits no specific fragmentation behavior. Conversion of arginine to its pyrimidine derivative provides cyclic derivatives suitable for the amino acid sequencing of polypeptides containing arginine⁹². Arginine reacts with malonaldehyde (yields 83–100 %) to form S-N-(2-pyrimidinyl)-L-ornithine.

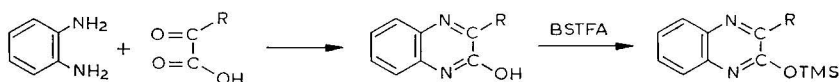


Benzoyl-L-arginine and malonaldehyde in ethanol as solvent produced simultaneous condensation of the guanidino group and formation of the ethyl ester without racemization. The derivative formed was volatile and should be suitable for GC and MS studies of polypeptides containing arginine residues⁹³. GC-MS was used to determine the trifluoroacetylated dimethylpyrimidyl derivatives of guanidinoacetic acid, β -guanidinopropionic acid, γ -guanidinobutyric acid, arginine and homoarginine in rat and bovine brain⁹⁴. The dimethylpyrimidyl derivatives of the guanidino group were prepared in pyridine–water (1:1) at pH 8.0–9.0 (adjusted by addition of sodium bicarbonate) by the addition of acetylacetone and refluxing at 100° for 10 h. For chro-

determined in plasma. A similar reaction with nitrous acid was used to determine dihydralazine in plasma with methylhydralazine as internal standard by reversed-phase HPLC⁹⁸. The limit of detection for dihydralazine at $\lambda = 230$ nm was 2.5 ng ml^{-1} of plasma. It has been suggested that the acidic conditions used in the derivative reaction could cause hydrolysis of acid-labile conjugate metabolites resulting in an elevated level for the free dihydralazine concentration⁹⁹. Lowering the pH of the solution to 3.0 for formation of the derivative should enable the real dihydralazine concentrations to be determined.

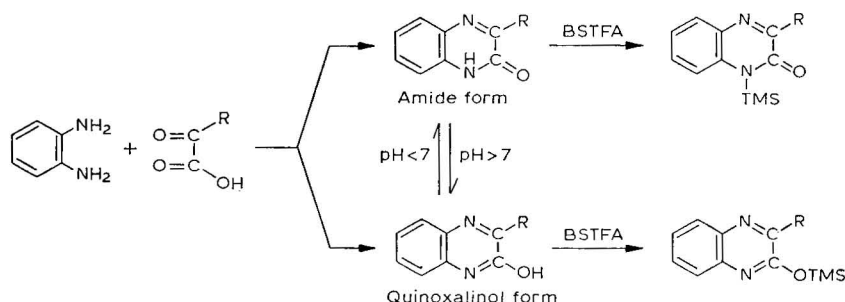
6. QUINOXALINOL DERIVATIVES OF α -KETO ACIDS

1,2-Diaminobenzene selectively reacts with α -keto acids in acid solution to form quinoxalinols which are thermally and hydrolytically stable cyclic derivatives.

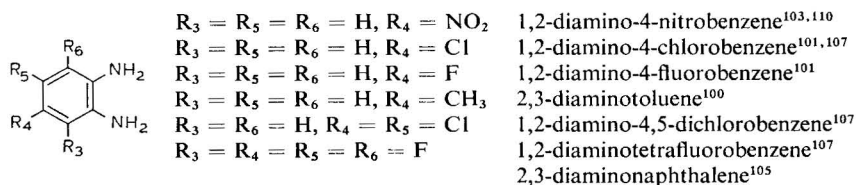


For gas chromatography, the derivatives are converted to their trimethylsilyl ethers. Optimum conditions for the reaction are a large excess of the diaminobenzene reagent in ethanol-acetic acid solution¹⁰⁰ or 2–4 *M* aqueous hydrochloric acid^{101,102} at elevated temperatures. The quinoxalinol reaction is very selective and no interference from acetic, oxalic, lactic, citric, 2-hydroxybutyric, acetoacetic acids, acetone, glucose, fructose, ascorbic acid or dehydroascorbic acid at levels encountered in biological fluids was observed¹⁰³. Under the conditions employed for quinoxalinol formation, the nitrite ion is converted into a benzotriazole derivative⁵⁴ and selenium forms a piaszelenole derivative¹⁰⁴. Only two α -keto acids can not be determined as their quinoxalinol derivatives¹⁰². Oxaloacetic acid (and its methyl ester, oxaloacetate) undergo decarboxylation in acid solution to form pyruvic acid which does not form a stable quinoxalinol derivative. At low pH, *o*-hydroxyphenylpyruvic acid forms a stable δ -lactone which does not react with the diaminobenzene reagent. An enzymatic method has been developed for the decomposition of oxaloacetate prior to the determination of α -keto acids in tissue samples¹⁰¹. The aromatic α -keto acids (phenylpyruvic, *o*- and *p*-hydroxyphenylpyruvic and indolpyruvic acids) are unstable to atmospheric oxidation. This may be suppressed by the addition of sodium hydrogen sulfide¹⁰⁰ or 2-mercaptoethanol¹⁰⁵ to the reaction mixture.

Still some confusion remains concerning the structure of the derivative formed with 1,2-diaminobenzene and its subsequent analysis by GC after silylation. Hoffman and Killinger¹⁰⁶ have assigned the amide form to the trimethylsilyl derivative based on IR and NMR evidence. Frigerio *et al.*¹⁰⁷ could find no evidence for significant amide absorption in the IR spectra of the derivatives they prepared and likewise, using Fourier transform GC-IR, Langenbeck *et al.*¹⁰⁸ also failed to detect any amide absorption. This coupled with the prominent loss of m/e 89 [$\cdot\text{OSi}(\text{CH}_3)_3$] in the mass spectrum of the quinoxalinol trimethylsilyl ether^{107–109} tends to favor the phenolic tautomer structure. Langenbeck *et al.*¹⁰⁹ has rationalized the evidence in terms of the pH of the silylation reaction solution which they claim favors quinoxalinol TMS ether derivative formation.



To effect changes in chromatographic resolution and to increase sensitivity to selective GC detectors, several analogues of 1,2-diaminobenzene have been used. These are summarized below,



The quinoxalinol derivatives of α -keto acids can be separated by paper chromatography^{100,101,103,110-112} and by TLC and column chromatography¹¹⁰. For the development of paper chromatograms, alcoholic solvents containing various amounts of aqueous ammonia were preferred^{100,103,112} as solvent mixtures of this type produced compact spots with the minimum of streaking. The use of fast-flow paper and spraying with sodium hydroxide prior to development gave improved chromatographic behaviour of the quinoxalinol derivatives¹⁰⁰. Quinoxalinol derivatives were detected by their fluorescence in long-wavelength UV light with a detection limit in the region of 0.05–0.02 $\mu\text{mole}^{100,101}$. For visual detection, the chromogenic reagent 1,2-diamino-4-nitrobenzene was introduced^{103,110}. The quinoxalinol derivatives were weakly yellow in color and faded rapidly when removed from the TLC tank unless sprayed with potassium hydroxide solution. For quantitative analysis, elution of the spots with alkaline ethanol and spectrophotometric measurement of the eluted solution gave the best results^{100,103}. Paper chromatography of the α -keto acid quinoxalinol derivatives has been used for their determination in *Penicillium chrysogenum*¹¹³, rat urine¹¹⁴ and human blood and urine^{100,103}.

Separation by GC of the quinoxalinol TMS ethers provides a rapid means of analysis giving complete separation of the α -keto acids of importance for studies of human metabolic dysfunction (with the exception of oxaloacetic and *o*-hydroxyphenylpyruvic acids)^{102,109}. The methylene unit (MU) values of these quinoxalinol derivatives on the three stationary phases OV-1, OV-17 and Dexsil are summarized in Table 6. On Dexsil 300 GC, palmitic acid (generally found in urine) and carboxyethylquinoxalinol (from α -ketoglutaric acid) are well separated, whereas they are eluted as a single peak on OV-1. Branched chain α -keto acids are determined on OV-1 because *n*-propylquinoxalinol (from α -keto valeric acid) and isobutyl-1-quinoxalinol (from α -keto- β -methylvaleric acid) are well separated on this phase but not on Dexsil¹⁰².

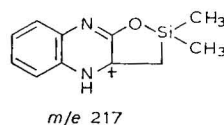
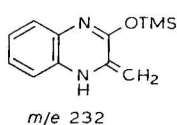
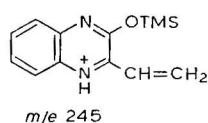
TABLE 6

METHYLENE UNIT VALUES OF TRIMETHYLSILYLQUINOXALINOLS

3% stationary phase loading on Gas-Chrom Q (100–120 mesh), nitrogen flow-rate 60 ml min⁻¹ and temperature programme from 50 to 180° at 2° min⁻¹.

<i>α</i> -Keto acid	<i>MU Value</i>		
	<i>OV-1</i>	<i>OV-17</i>	<i>Dexsil 300 GC</i>
Pyruvic	15.13	16.57	15.41
Ketobutyric	15.78	17.17	16.06
Ketoisovaleric	16.00	17.21	16.20
Ketovaleric	16.46	17.84	16.73
Keto- β -methyl- <i>n</i> -valeric	16.65	17.90	16.81
Ketoisocaproic	16.77	18.07	16.95
Ketooctanoic	19.23	20.68	19.50
Keto- γ -(methylthio)butyric	19.48	21.83	20.11
Ketoglutaric	20.48	22.28	20.76
Phenylpyruvic	20.63	23.24	21.26
Ketoadipic	21.48	23.34	21.81

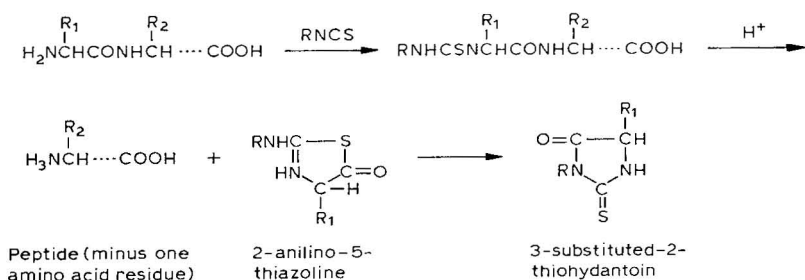
Using acetone deproteinization and cation-exchange chromatography as a sample pre-treatment method for biological fluids (urine, plasma, muscle tissue) as little as 0.005 mM of the α -keto acid quinoxalinol trimethylsilyl ether derivatives were determined with the flame ionization detector (FID)^{115,116}. This compares with previous detection limits of 0.1–0.15 mM obtained for α -keto acids in urine without preliminary separation by cation-exchange chromatography¹⁰². To improve the sensitivity and selectivity with which the quinoxalinol derivatives can be determined, the AFID¹¹⁴ and mass fragmentography^{98,104,105} have been used. No detection limit was given for the AFID (rubidium sulphate disk) but it was stated that a calibration curve could be established for the range 4–40 mg l⁻¹ of α -keto acids and this was adequate for the direct determination of α -keto acids in 50 ml of urine^{117–119}. This corresponds to a sensitivity enhancement over the FID of about 50-fold¹¹⁹. Using mass fragmentography, 30–100 pmoles of the α -keto acids could be determined depending on the ion selected for analysis and the ion current distribution for the quinoxalinol trimethylsilyl ether derivative^{102,108,109}. The technique is sufficiently sensitive for the determination of α -keto acids in 2.0-ml urine samples. The mass spectra of ten α -keto acids as their quinoxalinol TMS ether derivatives have been reported¹⁰⁸ and characteristic ions identified. One of the ions from the series *m/e* 217, 232 and 245 being present in nearly all derivatives studied were selected for mass fragmentography.



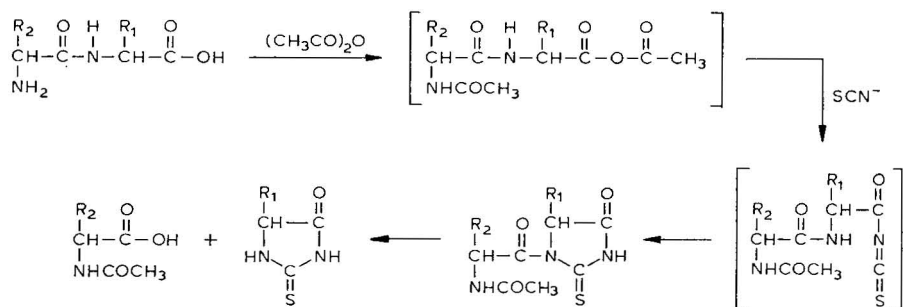
2,3-Diaminonaphthalene has been used for the selective analysis of phenylpyruvic acid in urine by reversed-phase HPLC¹⁰⁵. Other α -keto acids such as pyruvic, 2-oxobutyric, 2-oxoglutaric and 4-hydroxyphenylpyruvic acids did not interfere in the determination. Using UV absorption at $\lambda = 254$ nm the phenylpyruvic acid derivative could be easily detected below the μ g level of derivative injected on-column.

7. THIOHYDANTOIN DERIVATIVES OF AMINO ACIDS

The Edman degradation procedure is commonly used for the sequential analysis of the amino acid composition of proteins and peptides. The free amino group of the peptide is reacted with an organic isothiocyanate to form a thiocarbamoyl peptide derivative which is subsequently cleaved under acid conditions to liberate the 2-anilino-5-thiazolinone derivative and the original peptide less the terminal amino acid group. The 2-anilino-5-thiazoline derivative is rearranged under acid conditions to the more stable cyclic thiohydantoin derivative which is separated from the peptide and used to identify the terminal amino acid group by one of several chromatographic techniques. The whole procedure can be automated and sequential peptide analyzers are commercially available. The chemical steps in the procedure are shown schematically below:



Although less frequently applied, sequencing of peptides from the carboxylic acid end group can be achieved in a similar manner to that described for the amine end group. The procedure, shown below, was first described by Schlack and Kumpf¹²⁰ and developed as a method of sequencing by Cromwell and Stark¹²¹ and Yamashita¹²². The method is applicable to all polypeptides having a free carboxylic acid group and a free or monosubstituted α -amino group. The procedure described by Yamashita employs an ion-exchange resin to cleave the peptidyl thiohydantoin bond and does not promote cleavage of the parent peptide bonds observed sometimes with the acid conditions described by Cromwell and Stark.



A full discussion of the Edman procedure is beyond the scope of this review. Recent reviews by Edman¹²³, Niall¹²⁴, Rosmus and Deyl^{125,126} and Deyl¹²⁷ cover this topic adequately. The reviews by Rosmus and Deyl¹²⁵⁻¹²⁷ appeared in this journal

and to avoid unnecessary duplication only a brief summary of the chromatographic separation of thiohydantoin will be given here.

7.1. 2-Thiohydantoin

The 2-thiohydantoin derivatives of the amino acids can be identified by TLC on silica gel^{121,122,128} and polyamide sheets¹²⁹. Usually at least two solvent systems are necessary for the unequivocal identification of the protein amino acid derivatives on silica gel¹²¹. For the identification of thiohydantoin amino acid derivatives on polyamide plates, two-dimensional development of the TLC plate is employed with acetic acid–water (7:13) in the first direction and chloroform–95% ethanol–acetic acid (20:10:3) in the perpendicular direction. All common protein amino acid derivatives can be separated except for glutamine and glutamic acid (the latter may arise by hydrolysis of the former under the experimental conditions). The thiohydantoin strongly quench fluorescence in UV light and can be detected at the 50–100-nmole level on silica gel plates^{121,122} and at the 0.5-nmole level on polyamide sheets¹²⁹. The amino acid thiohydantoin derivatives produce a range of characteristic colors when sprayed with ninhydrin solution and this aids the confirmation of their identity¹²⁸.

The thiohydantoin derivatives of alanine, valine, leucine, isoleucine and methionine are sufficiently volatile to be separated by GC without derivatization¹²¹. Early attempts to prepare TMS and methyl derivatives of the remaining amino acid thiohydantoin resulted in the formation of doublet peaks on GC. Formation of the TMS derivatives in pyridine–BSTFA (1:1) at 50° for 10 min gave two peaks for glycine (mono- and bis-TMS derivatives), serine and threonine (side-chain dehydration) as well as causing the racemization of isoleucine to D-*allo*-isoleucine¹³⁰. With a mixture of ethyl acetate–BSA (1:1) and heating at 80° for 5 min doublet peaks were obtained for glycine, threonine, asparagine, carboxymethylcysteine and pyridylethylcysteine thiohydantoin¹²⁸. Fourteen TMS-thiohydantoin derivatives were separated and identified on a 4-ft. column of 10% SP-400 on Chromosorb W HP in less than 30 min. The derivatives of aspartic acid, methionine, glutamic acid, phenylalanine and asparagine were not resolved to baseline and the peaks for histidine, lysine and tyrosine were poorly separated. Mass spectra of the TMS-thiohydantoin amino acid derivatives have been recorded^{130,131}.

By using three isocratic solvent systems at 62°, sixteen thiohydantoin amino acid derivatives were identified by reversed-phase HPLC¹³². The derivatives of valine and tyrosine co-elute with a mobile phase of 0.01 *M* sodium acetate (pH 4.5) but are separated when the mobile phase contains 24% acetonitrile. Complete resolution of the hydrophobic (valine, isoleucine, leucine) and aromatic (phenylalanine, tryptophan) amino acid derivatives was obtained with a mobile phase consisting of 0.01 *M* sodium acetate (pH 4.5) and 10% acetonitrile.

7.2. 3-Methyl-2-thiohydantoin

The mobility (R_F values) of nineteen methylthiohydantoin (MTH) amino acid derivatives on polyamide sheets with two solvent systems has been described¹³³. Neither solvent was able to separate leucine and isoleucine MTH derivatives. 0.05–0.1 nmole of the MTH-amino acid derivatives could be detected by fluorescence quenching in UV light.

All the common protein MTH-amino acid derivatives except for aspartic acid,

arginine, serine, threonine, cysteic acid, S-carboxymethylcysteine and glutamic acid are sufficiently volatile to be separated by GC without derivatization¹³⁴. Methylene unit values for the stable MTH-amino acid derivatives are given in Table 7¹³⁷. A complete separation of all the MTH-amino acids of interest for protein sequencing has not been achieved by GC and the decomposition of some MTH derivatives produces products interfering with the identification of others (some MTH-amino acid derivatives decompose on metal columns)¹³⁶. To improve separation characteristics and to reduce column adsorption of the MTH-amino acids the trimethylsilyl derivatives have been prepared^{134,135,137-139}. Conditions employed for the formation of TMS-MTH-amino acid derivatives are BSTFA-acetonitrile (1:1.02) at 80° for 5 min¹³⁸, BSA-ethyl acetate (1:1) at 187° for 30 sec or 50° for 10 min¹³⁴, dissolution at room temperature in acetonitrile-BSA (3:1)¹³⁹ or acetonitrile-BSA (3:1) at 100° for 10 min¹³⁵. Of the common protein amino acids the MTH derivative of glycine was converted to the mono-TMS derivative, tryptophan was incompletely silylated and the TMS-MTH derivatives of aspartic acid, glutamine, ornithine, lysine and histidine were partly degraded on the GC column¹³⁸. Nineteen TMS-MTH-amino acid derivatives could be separated by temperature program analysis in less than 1 h¹³⁸. Eighteen TMS-MTH-amino acid derivatives were separated by temperature program analysis on OV-17 as stationary phase. The derivatives of phenylalanine and asparagine co-elute in this system¹³⁵. Retention index values for the TMS-MTH protein amino acids are given in Table 8. MS shows that silylation occurs on the thiohydantoin ring in all cases as well as on the side chain functional groups¹³⁹.

The FPD in the sulfur mode can be used to improve the selectivity and sensitivity of detection of the TMS-MTH-amino acid derivatives¹⁴⁰. Detection limits in the range 0.07–0.039 nmoles were obtained. The TMS-MTH derivatives of threonine,

TABLE 7

METHYLENE UNIT VALUES FOR THE MTH DERIVATIVES OF THE COMMON PROTEIN AMINO ACIDS ON TWO STATIONARY PHASES¹³⁷

6 ft. × 0.25 in. I.D. glass columns. Nitrogen flow-rate 90 ml min⁻¹.

<i>MTH-Amino acid</i>	<i>MU Value</i>	
	<i>5% SE-30</i>	<i>3% OV-17</i>
Glycine	14.63	19.08
Valine	15.12	18.68
Proline	15.49	19.95
Threonine	15.75	19.64
Alanine	15.81	20.82
Isoleucine	16.14	19.78
Leucine	16.26	19.81
Glutamic acid	17.80	23.70
Methionine	18.88	23.76
Phenylalanine	19.61	24.88
Asparagine	21.28	26.35
Glutamine	22.00	28.00
Lysine	23.07	29.98
Tyrosine	23.17	29.47
Histidine	23.78	30.16
Tryptophan	26.77	34.25

TABLE 8

RETENTION INDICES FOR THE TMS-MTH COMMON PROTEIN AMINO ACIDS ON TWO STATIONARY PHASES

<i>TMS-MTH-Amino acids</i>	<i>Column temperature (°C)</i>	<i>Retention index</i>	
		<i>SE-30*</i>	<i>OV-17**</i>
Alanine	140	1491	1724
Valine	140	1561	1757
S-Carboxymethylcysteine	140	1546	1773
Isoleucine	140	1649	1837
Leucine	140	1658	1857
Δ -Threonine	140	1582	1905
Proline	140	1543	1905
Glycine	140	1611	1764
Aspartic acid	180	1896	2140
Methionine	180	1912	2203
Glutamic acid	180	2016	2246
Phenylalanine	180	2005	2307
Asparagine	180	2016	2307
Glutamine	180	2154	2447
Tyrosine	220	2343	2616
Histidine	220	2298	2645
ϵ -MTC-Lysine	220	2326	2721
Tryptophan	220	2631	2926

* 175 \times 0.4 cm I.D. glass column packed with 1.70% SE-30 on Supelcoport (80–100 mesh).** 175 \times 0.4 cm I.D. glass column packed with 1.70% OV-17 on Supelcoport (80–100 mesh).

glycine, asparagine, glutamine, proline, histidine and ϵ -MTC-lysine gave low responses to the FPD when less than 2.5 nmoles were chromatographed.

Isolation of the thiazoline intermediate of asparagine and glutamine was used to distinguish these two amino acids from aspartic acid and glutamic acid which are formed under the acid conditions required for cyclization to the thiohydantoin derivative. The amino acids are identified by GC-MS after formation of their TMS derivatives¹⁴¹.

7.3. 3-Phenyl-2-thiohydantoin

Silica gel TLC has often been used for the identification of phenylthiohydantoin (PTH) amino acid derivatives. For the selection of useful solvent systems, the reviews mentioned at the beginning of this section should be consulted. Usually two solvent systems are necessary for the unequivocal identification of a PTH-amino acid. One solvent system is used to separate the polar PTH-amino acid derivatives and a second solvent system to separate derivatives with similar mobilities (e.g. leucine/isoleucine, valine/phenylalanine)¹⁴². Spraying the plate after development with ninhydrin produces characteristic colors for the different PTH-amino acid derivatives which can be used to aid identification¹⁴³. The use of micro silica gel TLC plates (6.3 \times 6.3 cm) has been recommended as providing an increase of 10–20-fold in the speed of analysis¹⁴⁴. It was also claimed that aluminium backed silica gel plates are superior to glass plates giving more compact spots and better resolution^{145,146}. Nearly all the PTH derivatives of the common protein amino acids can be identified in a

short time by two-dimensional development on polyamide layers. Twenty-one PTH-amino acid derivatives were identified with the solvent systems ethylene chloride-glacial acetic acid (90:16) and toluene-pentane-glacial acetic acid (60:30:20)¹⁴⁷. Leucine/isoleucine and methionine/methionine sulfone were not separated. Sixteen out of twenty PTH-amino acid derivatives were resolved using toluene-pentane-glacial acetic acid (60:30:35) and 35 % aqueous acetic acid as solvent systems¹⁴⁸. The relative mobility of the PTH derivatives was found to be very reproducible in this system and representative R_F values are summarized in Table 9. Twenty-four PTH-amino acid derivatives could be identified in less than 30 min with a two-dimensional development using toluene-pentane-acetic acid (60:30:16) and either 25 % aqueous acetic acid or 40 % aqueous pyridine-acetic acid (9:1) as the second solvent¹⁴⁹. The addition of a fluorescence indicator to the polyamide layer or developing solvent enabled 0.05–0.2 nmoles of the PTH-amino acid derivatives to be detected in UV light^{148,149}.

TABLE 9

R_F VALUES FOR THE COMMON PROTEIN AMINO ACIDS IN TWO SOLVENT SYSTEMS ON 5×5 cm POLYAMIDE SHEETS

PTH-Amino acid	R_F Value	
	Toluene-pentane-acetic acid (60:30:35)	35% Aqueous acetic acid
Alanine	0.63 ± 0.01	0.52 ± 0.02
Arginine	0.63 ± 0.01	0.95 ± 0.02
Asparagine	0.31 ± 0.02	0.63 ± 0.01
Aspartic acid	0.28 ± 0.02	0.46 ± 0.01
Cysteic acid	0.02 ± 0.00	0.13 ± 0.01
Glutamine	0.54 ± 0.01	0.60 ± 0.02
Glutamic acid	0.37 ± 0.02	0.49 ± 0.01
Glycine	0.56 ± 0.02	0.57 ± 0.01
Histidine	0.03 ± 0.01	0.95 ± 0.02
Isoleucine	0.82 ± 0.02	0.33 ± 0.01
Leucine	0.82 ± 0.02	0.33 ± 0.01
Lysine	0.45 ± 0.02	0.16 ± 0.00
Methionine	0.71 ± 0.03	0.38 ± 0.01
Phenylalanine	0.74 ± 0.03	0.28 ± 0.01
Proline	0.87 ± 0.03	0.51 ± 0.01
Serine	0.26 ± 0.02	0.61 ± 0.01
Threonine	0.39 ± 0.02	0.59 ± 0.01
Tryptophan	0.38 ± 0.02	0.16 ± 0.00
Tyrosine	0.18 ± 0.01	0.26 ± 0.01
Valine	0.79 ± 0.02	0.40 ± 0.01

The PTH-amino acid derivatives of alanine, glycine, valine, leucine, isoleucine, methionine, proline and phenylalanine are sufficiently volatile to be gas chromatographed without further derivatization¹⁵⁰. Other PTH-amino acid derivatives were either unstable or exhibit poor chromatographic properties. The formation of TMS derivatives improves the general chromatographic properties of the PTH-amino acids markedly^{150–152}. PTH-proline does not form a TMS derivative and PTH-lysine and PTH-arginine do not form TMS derivatives stable to GC. PTH-cystine and PTH-

cysteine form TMS derivatives which do not correspond to simple derivatives. The PTH derivatives of serine, threonine, tyrosine, glutamine, asparagine, histidine and tryptophan are stable to GC after formation of their TMS derivatives. The relative retention times of some PTH-amino acids and their TMS derivatives are summarized in Table 10¹⁵⁷. Combined GC-MS indicates that silylation occurs on the nitrogen atom of the hydantoin ring as well as the expected side-chain functional groups, the imidazole ring of histidine and the phenol group of tyrosine^{151,153}. The separation of all the common protein amino acid TMS-PTH derivatives on packed columns is difficult to achieve and usually some overlaps exist between different pairs of amino acid derivatives depending on the stationary phase selected for the separation. With the FPD (sulfur mode), nanogram amounts of the TMS-PTH amino acid derivatives were detected¹⁵².

TABLE 10

RELATIVE RETENTION TIMES OF PTH-AMINO ACIDS AND THEIR TMS DERIVATIVES ON SE-30¹⁵⁷

<i>Amino acid</i>	<i>PTH derivative (170°C)</i>	<i>TMS-PTH derivative</i>
Alanine	0.79	1.03
Glycine	0.94	1.05
Isoleucine	1.55	2.12
Leucine	1.65	1.98
Proline	1.38	1.38
Serine	0.89*	2.24
Threonine	0.97*	2.76
Valine	1.12	1.41
	(200°C)**	
Asparagine		5.53
Aspartic acid		3.41
Glutamine		8.25
Glutamic acid		4.91
Methionine	3.72	3.86
Phenylalanine	5.00	5.23
	(225°C)***	
Tyrosine	1.13	1.03
Histidine	1.27	0.98
Tryptophan	2.59	2.13

* Dehydration product.

** Relative to androstane.

*** Relative to cholestane.

Acetyl and trifluoroacetyl derivatives of PTH-amino acids have also been prepared to improve their separation properties¹⁵⁴⁻¹⁵⁶. The acetyl derivatives are considerably less volatile than the trifluoroacetyl derivatives but their chromatographic properties were considered to be superior¹⁵⁵. Unfortunately, a single set of experimental conditions could not be established for the formation of the acyl derivatives of all the PTH-amino acids important in protein sequencing^{154,155}.

The PTH-amino acid derivatives can be identified and quantified without chromatographic separation using chemical ionization MS with *tert*-butane as reagent gas¹⁵⁷. The PTH-amino acids are relatively strong bases and all have a stable

$[M+1]^+$ ion (except for lysine, SCM-cysteine and arginine) in their chemical ionization mass spectra which serves for their identification. Quantitation is achieved by using the d_5 -labelled PTH-amino acid as internal standard.

Recent years have witnessed an explosive growth in the application of HPLC to the identification and quantitation of the PTH-amino acids originating from the protein sequenator¹⁵⁸⁻¹⁷⁹. The advantages of HPLC compared to other techniques are its high speed, ease of automation, high sensitivity and the fact that further derivatization is not required prior to analysis. As little as 5 pmoles of PTH-amino acid derivatives can be detected with the UV detector¹⁶⁴. The PTH-amino acid derivatives differ widely in polarity and this has made the development of a single isocratic HPLC separation difficult. Chromatographic systems currently recommended for the separation of PTH-amino acids are summarized in Table 11. Many of the systems meet the requirement of being able to keep pace with the sequenator. Reversed-phase C_{18} columns with gradient elution using organically modified sodium acetate buffers are the most widely used separation method in HPLC for these derivatives.

7.4. Miscellaneous thiohydantoin

Pentafluorophenylisothiocyanate has been used instead of phenylisothiocyanate to form pentafluorophenylthiohydantoin (PFPTH) derivatives in the Edman procedure¹⁸⁰. The PFPTH-amino acids have good physical properties, can be identified by TLC and are significantly more volatile on GC than the PTH-amino acids.

p-Bromophenylisothiocyanate has been used in conjunction with MS to better identify PTH-amino acid derivatives without the aid of chromatographic separation¹⁸¹. The characteristic doublet for the bromine isotope peaks in the electron-impact mass spectra of the *p*-BrPTH derivatives simplifies identification based on the presence of a molecular ion.

p-Phenylazophenylisothiocyanate forms colored derivatives with amino acids obtained by the Edman procedure. All the *p*-phenylazophenylthiohydantoins (PAPTH) of the common protein amino acids can be identified within 30 min using silica gel TLC and two solvent systems¹⁸². Spraying the PAPTH amino acids with different reagents resulted in the development of characteristic colors which could be used to confirm the identity of a derivative when used in conjunction with R_F data¹⁸³.

The terminal carboxylic acid group of peptides can be sequenced by formation of its iminothydantoin derivative, a few of which have been separated by TLC and GC¹⁸⁴.

4-N,N-Dimethylaminoazobenzene-4'-isothiocyanate reacts with amino acids liberated during sequence analysis and the derivatives identified by two-dimensional TLC on polyamide sheets¹⁸⁵. The colored derivatives were detected down to the picomole level directly on the polyamide sheet.

8. CYCLIC SILICON-CONTAINING DERIVATIVES

Dimethyldichlorosilane (DMCS) in pyridine was used to form a cyclic siliconide derivative of 3 β -acetoxy-16 α ,17 α -dihydroxypregn-5-en-20-one and its mass spectrum recorded¹⁸⁶. DMCS in pyridine was also used to form a siliconide derivative of 3-methyl-18-hydroxylestradiol-17 β (the 17 α analogue does not form a derivative)¹⁸⁷. The principle problem in using DMCS as a reagent is that polyfunctional steroids con-

TABLE 11
CONDITIONS USED IN HPLC FOR THE SEPARATION OF PTH-AMINO ACIDS
FR = Flow-rate.

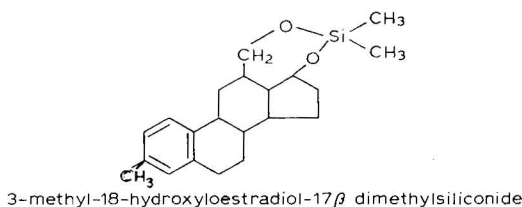
Stationary phase	Method of operation	Mobile phase	Comments on separation	Ref.
Adsorption 1.0 m × 1.8 mm I.D. 15–20 μm silica	Isocratic FR = 1.0 ml min ⁻¹ 25°	(a) C ₈ H ₁₆ -CHCl ₃ (1:1) (b) CHCl ₃ (c) CH ₃ OH-CHCl ₃ (3:97)	(a) Met, Phe, Val, Ile, Leu, Pro separated in 16 min (b) Ala, Gly, Tyr, Thr separated in 28 min (c) Asn, Gln, Ser separated in 12 min	158
50 cm × 0.3 mm I.D. Merckosorb SI 60	Isocratic FR = 1.65 ml min ⁻¹	(a) CH ₂ Cl ₂ -DMSO- <i>tert</i> -C ₄ H ₉ OH (100:0.8:8) (b) CH ₂ Cl ₂ -DMSO-H ₂ O (180:15:2) (c) CH ₂ Cl ₂ - <i>tert</i> -C ₄ H ₉ OH (1000:8)	(a) Pro, Leu, Ile, Val, Phe, Met, Ala, Trp, Gly separated in 7 min (b) Tyr, Lys, Thr, Gln, Asn, Asp separated in 7 min, Gly/Gln, Tyr/Thr poorly separated (c) Leu, Ile and 7 other PTH-amino acids separated in 9.5 min	159
50 cm × 0.2 cm I.D. Merckosorb SI 60	Isocratic FR = 0.5 ml min ⁻¹	CH ₂ Cl ₂ - <i>iso</i> -C ₃ H ₇ OH (1000:8)	Pro, Leu, Ile, Val, Phe, Met, Trp, Ala, Gly separated in 17 min	160
25 cm × 2.1 mm I.D. Zorbax Sil, 8 μm	Concave gradient FR = 0.6 ml min ⁻¹	C ₆ H ₁₄ -CH ₃ OH-C ₃ H ₇ OH (3980:9:11) to CH ₃ OH-C ₃ H ₇ OH (9:11)	All PTH-amino acids (except His, Arg) identified in 40 min. Gly/Thr, Lys/Tyr co-elute. Lys, Thr identified from by-products	161
30 cm × 3.9 mm I.D. Porasil, 10 μm	Concave gradient FR = 1.5 ml min ⁻¹	(a) A: <i>iso</i> -C ₈ H ₁₈ ; B: CH ₃ OH- <i>iso</i> -C ₃ H ₇ OH (9:11); 5 → 70% B (b) A: <i>iso</i> -C ₈ H ₁₈ -CHCl ₃ ; B: CH ₃ OH-C ₆ H ₁₈ -CHCl ₃ (15:42.5:42.5); 1 → 100% B	(a) Leu/Ile, Phe/Met, Glu/Asp co-elute. Val/Pro, Gly/Thr/Trp not separated to baseline. 20 min (b) Trp/Lys co-elute. Trp/Gly, Tyr/Thr, Met/Phe not separated to baseline. 20 min	162
Partition 2.5 cm × 2.1 mm I.D. Micropak-CN, 10 μm	Step gradient FR = 0.83 ml min ⁻¹	A: C ₆ H ₁₄ B: CH ₂ Cl ₂ - <i>iso</i> -C ₃ H ₇ OH (1:1)	17 PTH-amino acids separated. Poor column efficiency. Many overlaps. 40 min	163

(Continued on p. 128)

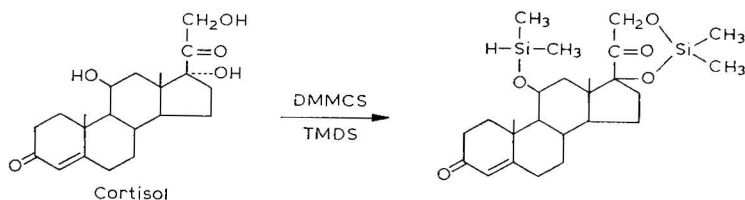
TABLE 11 (continued)

Stationary phase	Method of operation	Mobile phase	Comments on separation	Ref.
1 m × 2 mm I.D. Permaphase ETH, 30 μm	(a) Isocratic (b) Exponential concave gradient	(a) 0.01 M NaOAc (b) A: 0.01 M NaOAc; B: CH ₃ CN; 10% B min ⁻¹	(a) His/Arg separated in 12 min. 5 other non-polar derivatives separated in 20 min (b) Ser/Thr/His/Arg co-elute. Tyr/ Met, Trp/Lys, Gln/Asn poorly separated. 40 min	164
50 cm × 2.1 mm I.D. Permaphase ETH	Linear gradient	A: H ₂ O; B: CH ₃ CN; A → 30% B at 2% min ⁻¹	12 PTH-amino acids separated. 17 polar PTH derivatives separated C ₁₈	
Reversed-phase 3 ft. × 2.0 mm I.D. Corasil C ₁₈	Linear gradient FR = 0.8–1.2 ml min ⁻¹	A: 0.01 M NaOAc (pH 3–8)–CH ₃ CN (95:5); B: CH ₃ CN; 2% B min ⁻¹	Few PTH-amino acids separated to baseline. 30 min	165
1 m × 4.0 mm I.D. Corasil C ₁₈	Step gradient FR = 1.0–1.2 ml min ⁻¹	H ₂ O–CH ₃ CN–iso-C ₃ H ₇ OH (100:1.5:1) to (15:1.5:1)	Poor column efficiency. Many overlaps	166
60 cm × 4.0 mm I.D. Bondapak C ₁₈	3 gradient programs	A: 0.01 M NaOAc (pH 4.0)–CH ₃ CN (9:1); B: 0.01 M NaOAc (pH 4.0)– CH ₃ CN (1:9)	20 PTH-amino acids identified by a choice of 3 systems using two buffers	167
25 cm × 2.0 mm I.D. Zorbax ODS, 5–6 μm	Linear gradient FR = 0.5 ml min ⁻¹ 50°	A: 0.01 M NaOAc (pH 5.0)–CH ₃ CN (95:5); B: CH ₃ CN; 1% B min ⁻¹	All common PTH-amino acids separated except Gly/Gln, Met/Val which co-elute. Phe/Leu/Ile poorly separated	164
25 cm × 4.6 mm I.D. LiChrosorb C ₁₈	3 gradient programs	A: 0.1 M NaOAc (pH 4.6) at 82°; B: CH ₃ CN at 47°	All common PTH-amino acids separated in 3 programmed gradient runs	168
25 cm × 4.6 mm I.D. Partisil ODS, 10 μm	Isocratic FR = 2–3 ml min ⁻¹	0.01 M NaOAc (pH 4.0) with 10, 20 and 30% CH ₃ CN	21 PTH-amino acids separated in 3 isocratic runs	169
25 cm × 4.6 mm I.D. Zorbax ODS	Gradient FR = 1.0 ml min ⁻¹	A: 0.01 M NaOAc (pH 4.5); B: CH ₃ CN	Under isocratic conditions A–B (42:58) all PTH-amino acids except Ser/Gln which co-elute are separated in 15 min. 20-PTH-amino acids separated by gradient elution. 20 min. Ser, Gln separated in a separate system.	170

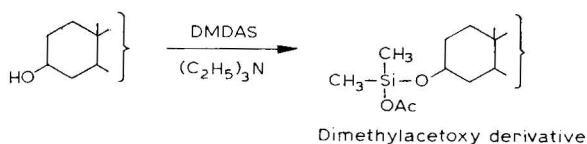
30 cm × 3.9 mm I.D. μBondapak C ₁₈ , 10 μm	Step gradient	A: 0.005 M NaOAc (pH 5.3)-acetone (77:23) + 20 μl CH ₃ OH; B: as above, 44% CH ₃ OH C: as A, 22% CH ₃ OH-5% CH ₃ COOH	17 PTH-amino acids separated in A → 100% B. Val/Met, Phe/Ile co-elute. 16 min Solvent C separates Cys, His, Arg in 30 min isocratically	171
30 cm × 44 mm I.D. Bondapak C ₁₈	Linear gradient FR = 2.5 ml min ⁻¹	A: H ₂ O-CH ₃ OH-CH ₃ CO ₂ H (900:100:2.5) + 50 μl (CH ₃) ₂ CO; B: H ₂ O-CH ₃ OH-CH ₃ CO ₂ H (100:900:0.25); 95% A + 5% B to 55% A + 45% B	21 PTH-amino acids tested. Val/Met co-elute. Leu/Ile/Phe poorly separated. 20 min	172
2 × [15 cm × 3.9 mm I.D.] LiChrosorb RP-8, 5 μm	Isocratic FR = 0.9 ml min ⁻¹ 50°	0.01 M NaOAc (pH 4.6) (4:1)	21 PTH-amino acids separated. Met/Pro co-elute	173
30 cm × 4.0 mm I.D. Bondapak C ₁₈	Concave gradient + Isocratic period	A: 0.01 M NaOAc (pH 4.0)-CH ₃ OH (9:1); B: 0.01 M NaOAc (pH 4.0)-CH ₃ OH (1:9); 5% B → 40% B	All common PTH-amino acids separated except Met/Val. 32 min	174
30 cm × 4.0 mm I.D. μBondapak C ₁₈	4 gradient programs used	A: 0.01 M NaOAc (pH 4.0)-CH ₃ CN (9:1); B: as above (1:9)	(a) Ala, Tyr, Val, Ile, Lys separated in 20 min. (b) Asp, Asn, Ser, Thr, Gly, Gln, S-CMC separated in 15 min (c) Pro/Met, Trp/Phe separated isocratically (d) Cys, His, Arg separated in 8.0 min. 22 PTH-amino acids can be identified with 2 injections	175
30 cm × 4.0 mm I.D. Bondapak C ₁₈	Concave gradient FR = 4.0 ml min ⁻¹	A: 8.0 mM Diethylenetetramine + 20.0 mM trichloroacetic acid pH 4.2; B: as above + 60% CH ₃ CN	Trp/Phe/Leu and Pro/Val co-elute. Only a few separations to baseline. 40 min	176
<i>Bonded peptide stationary phases</i> 25 cm × 3.8 mm I.D. L-Val-L-Ala-L-Ser bonded onto silica CT	Isocratic	1% Citric acid-water pH 2.5	15 PTH-amino acids separated well enough for identification purposes	177
25 cm × 2.1 mm I.D. L-Val-L-Phe-L-Val bonded onto Partisil 10 0.26 mmole g ⁻¹ peptide	Isocratic	1% Aqueous citric acid (pH 2.5)-CH ₃ OH (20:1)	11 PTH-amino acids separated with good selectivity but column efficiency is poor	178



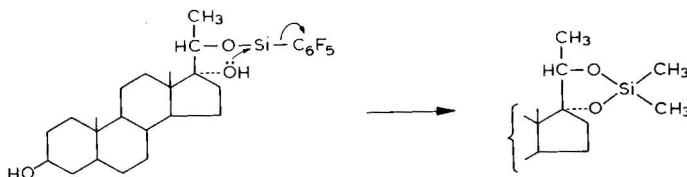
taining isolated functional groups (ketones as well as hydroxyl groups) react to various extents yielding thermally unstable products. Corticosteroids with an 11 β -OH group gave unstable products due to partial reaction of the hydroxyl group with DMCS¹⁸⁸. The siliconide derivative of the corticosteroid side chain was very moisture sensitive and was formed in about 40% yield when DMCS pyridine was used as the reaction medium. For corticosteroids with isolated functional groups (*e.g.*, cortisol) a mixed derivative has been prepared using dimethylmethoxychlorosilane (DMMCS) to form the side chain siliconide derivative and to simultaneously catalyze the reaction of 1,1,3,3-tetramethyldisilazane (TMDS) with the hindered 11 β -OH group¹⁸⁸. The yield



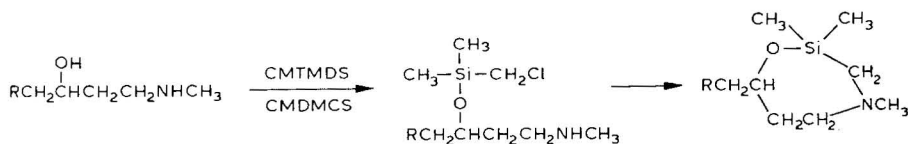
of mixed derivative was about 10% and the reaction is limited to steroids with hindered hydroxyl groups. Steroids with unhindered hydroxyl groups such as 3 α ,17 α ,21-trihydroxy-5 β -pregnane-11,20-dione react with the mixed reagent to form multiple products unsuitable for GC. To avoid this problem, dimethyldiacetoxysilane (DMDAS) was introduced¹⁸⁹. This reagent forms identical siliconide derivatives with diols and with isolated hydroxyl groups forms thermally stable dimethylacetoxysilane derivatives. The DMDAS reagent has moderate silyl donor power, reacting smoothly with unhindered hydroxyl groups in the presence of base catalysis but not significantly with 11 β and more sterically hindered hydroxyl groups. Reactions are carried out in dilute solution to minimize the formation of derivatives with silyl bridges (the diacetoxysilane reacting with two steroid molecules). The cyclic dimethylsiliconide derivatives were formed in 75–95% yield with *cis*-diols and steroids with the dihydroxyacetone side chain. In a separate study, dimethylsiliconides were formed satisfactorily with steroids containing a dihydroxyacetone side chain and 17 α ,20-diols but not from 20,21-diols¹⁹⁰. Both the reagent DMDAS and the derivatives were extremely susceptible to hydrolysis.



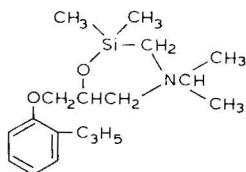
A dimethylsiliconide derivative was formed as an unexpected product from the reaction of flophemesylamine with 5 β -pregnane-17 α ,20-diol¹⁹¹. Presumably the bulky flophemesyl reagent reacts preferentially with the exposed 20-hydroxyl group and the dimethylsiliconide derivative is formed by nucleophilic attack of the 17 α -OH group at the silicon center with expulsion of pentafluorobenzene.



β - and γ -hydroxylated primary, secondary, tertiary and quaternary amines and possibly some enolizable ketone groups react with a mixture of 1,3-bis(chloromethyl)-1,1,3,3-tetramethyldisilazane (CMTMDS) and chloromethyldimethylchlorosilane (CMDMCS) to form cyclic derivatives¹⁹². The reaction takes place smoothly at room temperature or upon heating to 60° for 5–15 min. Under these conditions the amine



group is not derivatized and reaction initially takes place at the hydroxyl group followed by cyclization in solution involving elimination of chlorine as hydrogen chloride from the chloromethyl group. The reaction with phenacylamine (β -keto amine) was incomplete and although phenylalanine gave a cyclic derivative, other α -amino acids (tryptophan, proline) gave poor results. β -Hydroxy quaternary amino compounds may not react to completion and produce by-products on GC. The cyclic silicon derivatives have good GC properties and their mass spectra were characterized by prominent molecular ions and diagnostically informative fragmentation pathways. The metabolism of the β -adrenoceptor antagonist drug, alprenolol in man and dog was followed by formation of a cyclic silicon derivative of the side chain using the method described above¹⁹³. The low level of drug and its metabolites in biological fluids were determined by mass fragmentography.



Cyclic Dimethylsilyl Derivative of Alprenolol

The dimethylsilyl derivative was found to be stable to enzymatic hydrolysis and to the conditions necessary for forming TMS derivatives of hydroxylated metabolites with trimethylsilylimidazole. One metabolite of alprenolol, resulting from hydroxylation of the allylic substituent to a 1,2-diol, gave the normal bis(chloromethyldimethylsilyl)

ether and not the cyclic dimethylsilyl derivative under the usual reaction conditions for the formation of the cyclic derivative. Compounds which have been separated by GC as their cyclic silicon derivatives are summarized in Table 12.

TABLE 12

COMPOUNDS FORMING CYCLIC SILICON-CONTAINING DERIVATIVES USED IN GC

<i>Compound</i>	<i>Reagents</i>	<i>Comments</i>	<i>Ref.</i>
3 β -Acetoxy-16 α ,17 α -dihydroxy-pregn-5-en-20-one	DMCS	MS	186
Cortisone	DMCS	GC	188
	DMDAS	GC	189
Cortisol	DMMCS-TMDS	GC, MS	188
	DMDAS	GC	189
Tetrahydrocortisol	DMDAS	GC	189
Betamethasone	DMMCS-TMDS	GC, MS	188
	DMDAS	GC	189
3-Methyl-18-hydroxyoestradiol	DMCS	GC	187
17 α ,21-Dihydroxypregn-4-ene-3,20-dione	DMDAS	I_{OV-1} , MS	190
		I_{dex} .	
5 β -Pregnane-3 α ,17 α ,20 β -triol	DMDAS	I_{OV-1} , MS	190
		I_{dex} .	
	Flophemesylamine	GC, MS	191
Ephedrine	CMDMCS-CMTMDS	GC, MS	192
1-Phenyl-2-methylaminoethanol	CMDMCS-CMTMDS	GC, MS	192
Terbutaline*	CMDMCS-CMTMDS	GC	192
Propanolol**	CMDMCS-CMTMDS	GC, MS	192
Isoprenaline***	CMDMCS-CMTMDS	GC, MS	192
Aprobit [§]	CMDMCS-CMTMDS	GC, MS	192
Phenacylamine	CMDMCS-CMTMDS	GC	192
Phenylalanine	CMDMCS-CMTMDS	GC, MS	192
Clobutinol ^{§§}	CMDMCS-CMTMDS	GC, MS	192
Alprenolol ^{§§§}	CMDMCS-CMTMDS	GC, MS	193
4-Hydroxy-alprenolol	CMDMCS-CMTMDS	GC, MS	193
1'-Hydroxy-alprenolol	CMDMCS-CMTMDS	GC, MS	193
2',3'-Dihydroxy-alprenolol	CMDMCS-CMTMDS	GC, MS	193

* 2-*tert.*-Butylamino-1-(3,5-dihydroxyphenyl)ethanol.

** 1-Isopropylamino-3-(1-naphthyloxy)-2-propanol.

*** 1-(3,4-Dihydroxyphenyl)-2-isopropylaminoethanol.

§ 10-[2-(2-Hydroxyethyl)dimethylamino) propyl]-phenothiazine chloride.

§§ 4-Dimethylamino-1-(4-chlorophenyl)-2,3-dimethyl-2-butanol.

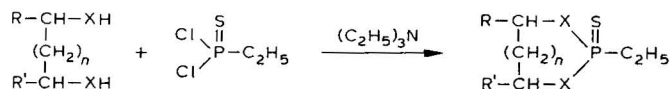
§§§ 1-Isopropylamino-3-(2-allylphenoxy)-2-propanol.

The reaction of organotrialkoxysilanes with trialkanolamines results in the formation of C-substituted silatranes (2,8,9-trioxa-5-aza-1-silatricyclo[3,3,3^{1,5}]-undecanes)¹⁹⁴. These are polar intramolecular complexes with a transannular N→Si bond. Although of high boiling point, they are sufficiently stable to be separated by GC.

9. CYCLIC ETHYLPHOSPHONOTHIOIC DERIVATIVES

Ethylphosphonothioic dichloride (EPTD) reacts with bifunctional compounds

containing OH, NH₂ and CO₂H groups in the presence of triethylamine to form cyclic ethylphosphonothioic derivatives according to the following equation¹⁹⁵:



Stable derivatives were also formed by *ortho* substituted (but not *meta* and *para*) bi-functional aromatic compounds and enolizable diketones. Dicarboxylic acids, α -hydroxyacids or aliphatic bifunctional compounds in which $n \geq 2$ did not form derivatives stable to GC. Although the rate of reaction depends on the steric arrangement and the chemical nature of the bifunctional group (*ortho* substituted aromatic compounds react slower than their aliphatic equivalent), all reactions studied were complete when 1.0 ml of a 10 mmolar solution of the bifunctional compound in acetonitrile, 15 μ l of ethylphosphonothioic dichloride and 33 μ l of triethylamine were heated at 80° for 30 to 45 min in a nitrogen atmosphere. Retention index data for some representative cyclic ethylphosphonothioic derivatives are given in Table 13. The second derivative peaks formed with phenyl-1,2-ethanediol and *cis*-1,2-cyclohexanediol are most probably due to the formation of geometric isomers based on mass spectral evidence. The EPTD derivatives can be determined with high selectivity at trace levels using selective GC detectors. Detection limits obtained with several detectors for a derivative of pinacol are summarized in Table 14. The NPD can be used to determine picogram quantities of the EPTD derivatives. The mass spectra of the EPTD derivatives are characterized by a prominent molecular ion which fragments to produce stable phosphorus-containing daughter ions constituting a considerable amount of the total ion current of the mass spectrum. The base peak in many spectra was formed by the loss of the elements of ethylsulfide from the molecular ion. Analysis of the mass spectra of the diol derivatives indicated the presence of characteristic ions useful for their identification.

TABLE 13

RETENTION INDEX VALUES FOR THE CYCLIC ETHYLPHOSPHONOTHIOIC DERIVATIVES OF BIFUNCTIONAL COMPOUNDS

90 \times 0.2 cm I.D. nickel column packed with 1% OV-17 on Gas-Chrom Q (100–120 mesh). Nitrogen flow-rate = 30 ml min⁻¹.

Cyclic ethylphosphonothioic derivative	Retention index (<i>I</i> _{OV-17})		Column temperature (°C)
	1st Peak	2nd Peak	
Ethylene glycol	1563	—	110
1,3-Propanediol	1568	—	110
Pinacol	1639	—	110
Catechol	1733	—	130
3-Amino-1-propanol	1788	—	130
1,3-Propanediamine	1968	—	150
<i>cis</i> -1,2-Cyclohexanediol	1961	2020	150
<i>o</i> -Aminophenol	2042	—	150
Phenyl-1,2-ethanediol	2185	2221	170
<i>o</i> -Phenylenediamine	2385	—	170

TABLE 14

THE MINIMUM DETECTABLE QUANTITY (MDQ) OF THE EPTD DERIVATIVE OF PINACOL

Detector	Pinacol (ng)	$P (g \text{ sec}^{-1})$ ($\times 10^{-15}$)	$S (g \text{ sec}^{-1})$ ($\times 10^{-10}$)
Nitrogen-phosphorus detector	0.002	2.0	—
Flame photometric detector (P mode)	0.5	2000	—
Flame photometric detector (S mode)	1.0	—	2.0
Electron-capture detector	10.0	—	—

Ethyldichlorothiophosphate did not yield derivatives stable to GC under the reaction conditions used to prepare EPTD derivatives¹⁹⁵.

10. CYCLIC BORONIC ESTERS

Since their introduction about twelve years ago by Brooks and Watson²⁰⁹, the organic boronic acids have become well established as the most useful reagents for the chromatographic analysis of bifunctional compounds. They owe their popularity to their ability to react with a wide range of polar functional groups under mild conditions to give stable derivatives with good GC and MS properties. Recently, Poole *et al.*²⁸² have introduced several boronic acids with electron-capturing properties to extend the range of application of the boronic acids to the realm of trace analysis. The combination of the selective reaction of the boronic acid group, the separating power of the gas chromatograph and the high sensitivity and selectivity of the electron-capture detector promises a new vista for trace analysis of bifunctional compounds in complex mixtures with the minimum of sample manipulation.

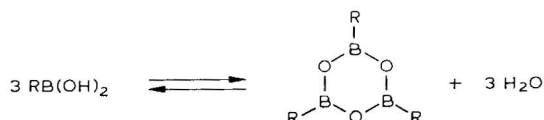
10.1. General chemistry of boronic acids

The synthesis and properties of the boronic acids have been reviewed from the point of view of their basic chemistry and reactions^{196–198}. A complete list of all boronic acids prepared up to about 1974 is available¹⁹⁸. Boron forms two types of organo-boron acids, the boronic acids and the borinic acids of general structure shown below:

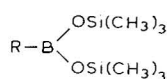


The borinic acids have not been shown to have any analytical uses and will not be considered further.

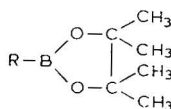
The boronic acids are very readily dehydrated to the trimeric cyclic anhydrides (boroxines) under mild conditions (*e.g.*, recrystallization). Most commercial samples



of boronic acids contain a variable amount of the anhydride. This is no disadvantage from the chromatographic point of view as the anhydride reacts as easily as the acid. The anhydride has good GC properties and is formed in the injection port when a solution containing a boronic acid is injected into the gas chromatograph. The anhydride is also formed when a boronic acid is heated in a melting point apparatus. Generally speaking, the determination of the melting point is not a good method of determining the purity of a boronic acid as the value obtained is dependent on the method and rate of heating. Complete dehydration of the acid by heating *in vacuo* over phosphorus pentoxide enables an accurate value for the anhydride to be obtained which can be used to characterize the original acid. The preparation of the highly crystalline and sharp melting diethanolamine derivatives are a more convenient method of characterization^{199,200}. In a chromatographic laboratory, it is easier to characterize a boronic acid or to identify impurities in a sample of boronic acid by chromatographic techniques. On-column conversion of the boronic acid to the anhydride is too variable for quantitative analysis and the separation of boronic acids by TLC is difficult as the mobility of the acid is dominated by the boronic acid function and little influenced by the organic substituent. Boronic acids form bis-TMS esters which have good GC properties and characteristic mass spectra²⁰¹. The molecular ions are generally weak but a prominent $[M-CH_3]^+$ ion can be used to identify the molecular weight. The bis-TMS ester derivatives are very hydrolytically unstable which makes their manipulation difficult. Pinacol can be used as a reagent to characterize boronic acids²⁰². This has the advantage of producing crystalline derivatives in quantitative yield (with aromatic boronic acids) which are hydrolytically stable and can be submitted to TLC or HPLC.



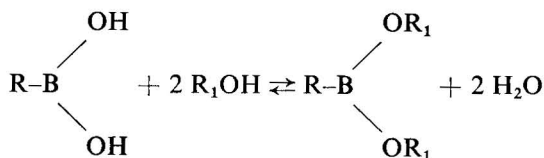
Boronic acid
bis-TMS ester



Pinacol boronate

The pinacol boronates are volatile with good peak shape on GC and their mass spectra are distinguished by a strong molecular ion and characteristic modes of fragmentation reflecting the nature of the organic function of the boronic acid.

Boronic acids react with monofunctional compounds such as alcohols to form dialkyl boronate derivatives. These derivatives are very hydrolytically unstable

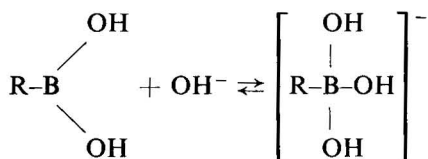


and decompose slowly in moist air. The dialkyl boronate derivatives are often employed to purify crude preparations of boronic acids where use is made of the easy reversibility of the equilibrium reactions; first to form the alkyl boronates which can be distilled and then the original boronic acid is recovered by hydrolysis. The dialkyl boronate derivatives are not usually stable to GC.

The aromatic boronic acids are air stable. The alkylboronic acids (but not their sodium salts) are slowly oxidized by dry air to the orthoboric acid which is moisture sensitive^{203,204}. The rate of reaction is influenced by the electron



releasing power of the alkyl substituent. For the butaneboronic acids the stability order is *tert.*-butane < isobutane < *n*-butane. The rate of oxidation is considerably reduced in the presence of moisture and for this reason commercial samples of alkyl boronic acids are supplied as an aqueous slurry and should be stored in a refrigerator. This does not present any problems in their use as derivatizing reagents, as the amount of water required to inhibit the autoxidation reaction is small and for many applications the acid can be used in this form. If it is necessary to remove all the water, then small portions of the acid can be dried on filter paper immediately before use or the slurry can be added to a water scavenger such as 2,2-dimethoxypropane which may be used as the solvent for the derivative reaction. Alternatively the boronic acid can be dissolved in a solvent containing a drying agent (*e.g.*, molecular sieves) which absorbs the water.



Boronic acids behave as acids in the Lewis' sense with the formation of a tetra-valent boron atom. Benzeneboronic acid ($\text{p}K$ 8.86) is three times as strong an acid as boric acid²⁰⁵ and approximately ten times as strong as *n*-butaneboronic acid²⁰⁶. The substitution of electronegative groups into the benzene ring of benzeneboronic acid considerably increases the acid strength whereas alkyl groups decrease the acidity to a much smaller extent. A theoretical interpretation of the effect of substituents on the acid strength of the boronic acids has been given^{207,208}.

10.2. The formation of boronate derivatives — An overview

The boronate derivatives enjoy widespread use for the analysis of bifunctional compounds by GC. Their dominant position in this area of chromatography is a consequence of their broad range of application, ease of reaction, good thermal and GC properties and their useful mass spectral features. Disadvantages include the poor hydrolytic stability exhibited by many derivatives and the ease of solvolysis observed in multiple derivatization procedures in which the boronate group may be partially or completely displaced when the selective reaction of a remote functional group is required to improve chromatographic properties. Boronate derivatives can be prepared from compounds having two functional groups in close proximity such as alkyl 1,2-diols, 1,3-diols, 1,4-diols, 1,2-enediols, 1,2-hydroxyacids, 1,3-hydroxyacids, 1,2-hydroxyamines, 1,3-hydroxyamines and aromatic compounds with *ortho* substituted phenol, amine and carboxylic acid groups. Many examples of bifunctional

compounds of this type are to be found among the physiologically important classes of compounds such as the steroids, carbohydrates, nucleosides, lipids, catecholamines, prostaglandins etc. Mild conditions are usually sufficient for derivative formation and a typical reaction involves mixing of the boronic acid and substrate in an anhydrous solvent at room temperature for a short time (1.0 min to 0.5 h). In some cases excess boronic acid may be required to force the equilibrium reaction to completion and for those derivatives which are exceptionally moisture sensitive a means of removing water produced in the reaction is required (*e.g.*, molecular sieves can be added to the reaction medium, 2,2-dimethoxypropane can be added as a water scavenger or periodic azeotropic evaporation with benzene or dichloromethane can be used). Direct injection into the gas chromatograph of boronate derivatives with remote unprotected polar functional groups in the presence of excess boronic acid invariably results in poor chromatographic performance exemplified by tailing peaks of reduced peak height. Sequential derivatization of the various functional groups is required in this case and special attention has to be paid to the possibility that strong reaction conditions could result in loss of the boronate group.

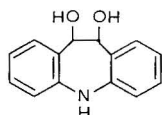
The boronic acids, methaneboronic acid, butaneboronic acid, *tert.*-butaneboronic acid, cyclohexaneboronic acid and benzeneboronic acid have all been used to prepare derivatives for gas chromatography. The cyclohexaneboronates and benzeneboronates have long retention times by comparison to the other boronate derivatives which can be inconvenient for the analysis of poly-bifunctional or high-molecular-weight compounds. The *tert.*-butaneboronates are surprisingly volatile on silicone stationary phases of low polarity with retention times closer to those of the methaneboronates than the *n*-butaneboronates. Unfortunately the reagent and derivatives have poor hydrolytic and air stability which limits their practical use. The methaneboronates are very volatile and the small molecular weight increment formed by derivatization is useful in the MS of high-molecular-weight compounds. The butaneboronate derivatives provide a convenient compromise between volatility and stability making them the most studied derivatives. The stability of the boronate derivatives to TLC and other hydrolysis conditions is variable depending both on local stereochemistry of the bifunctional group and the individual boronic acid used to prepare the derivative.

The boronate derivatives have useful mass spectral properties with prominent molecular ions or quasi $[M+1]$ molecular ions in the case of chemical ionization mass spectrometry. The boronate group is not strongly directing in influencing the mode of fragmentation as charge localization invariably occurs at a center remote from the boronate group due to the electrophilic character of the boron atom. This has the advantage that the abundant ions in the mass spectrum are characteristic of the parent molecule and not the derivatizing reagent. The natural isotope abundance of boron ($^{10}\text{B} : ^{11}\text{B} = 1:4.2$) aids the identification of boron containing fragments in the low-resolution mass spectra of the boronate derivatives. The boron-isotope distribution is a disadvantage when the mass spectrometer is operated as a single ion gas chromatographic detector, as the ion current carried by the boron containing fragment is divided in the same ratio as the isotope distribution with a consequent reduction in sensitivity.

In the following sections, the usefulness of the boronate derivatives is exemplified by considering their applications under a series of headings representing different types of functional groups and classes of biologically important substances. At the

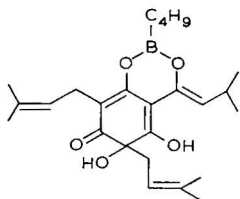
end of each section, all boronate derivatives are collated into a single table for rapid identification of literature sources to particular compounds. The mass spectra of many boronate derivatives have been recorded but as the principal modes of fragmentation observed are more characteristic of the parent molecule than the boronate group *per se*, reference is given to the availability of mass spectral information in the above mentioned tables and is not specifically reviewed here.

10.2.1. Diols and ketols. Diols and ketols react rapidly in solution with boronic acids to form cyclic boronate derivatives with good GC properties. The reaction is specific for *cis*-diols and can be used to distinguish between *cis*- and *trans*-diols by GC with a sample requirement of a few μg ^{209,210}. Some diols such as 1,3-propanediol and indane-*cis*-1,2-diol form particularly stable boronate derivatives which when added in excess to other boronate diol derivatives result in displacement of the original diol from the derivative which can then be recovered^{209,211}. Although the boronate derivatives are generally prepared in solution, they can also be prepared by co-injection of the diol and excess boronic acid into the gas chromatograph in high or quantitative yield. This approach was used to form butaneboronate derivatives for the mass spectral identification of *cis*-diols formed by the metabolism of drugs containing an epoxide ring (*e.g.*, carbamazepine, iminostilbene, cytenamide)^{212–215}. The determination of the activity of microsomal styrene monooxygenase (epoxide synthase and epoxide hydrolase using styrene or styrene epoxide as substrate was determined by GC of the phenylethyleneglycol produced after conversion to its butaneboronate²¹⁶.



Iminostilbene-10,11-dihydrodiol

Shaw²¹⁷ used butaneboronic acid to stabilize the β -hydroxyketol group of the hop bitter acids (humulone, cohumulone, lupulone and colupulone found in beer) and separated the products by GC. The butaneboronate derivatives were said to have useful mass spectral properties for the identification of the isomeric acids. Verzele *et al.*²¹⁸ found the butaneboronic and benzeneboronic acid derivatives to be of little value for the separation of this class of compound and recommended that the TMS ethers should be used.



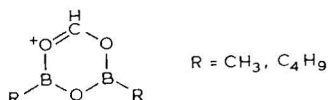
Butaneboronate of Humulone

Olefins can be selectively oxidized with osmium tetroxide to the *cis*-diols. Formation of the benzeneboronate and characterization of the derivatives by GC with electron-impact and chemical-ionization MS enabled the original position of the double bond to be established²¹⁹. By capillary column GC, a 35-component mix-

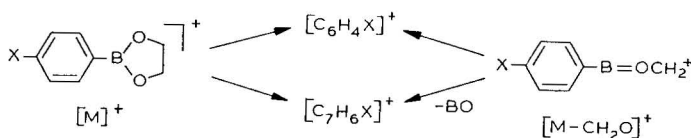
ture of C_5 – C_9 monoalkenes was separated after specific oxidation as their benzeneboronate derivatives. The chemical-ionization mass spectra of the benzeneboronates is characterized by a prominent quasi $[M+1]^+$ molecular ion which serves to identify the molecular weight. The electron-impact mass spectra show a characteristic α -cleavage reaction to produce an abundant fragment $[M-R]^+$ identifying the substituent attached to the original double bond.



The methyl esters of mono-unsaturated fatty acids after osmium tetroxide oxidation and formation of the benzeneboronate or butaneboronate derivatives show a similar ion due to α -cleavage⁶⁴. The di-unsaturated and tri-unsaturated fatty acid methyl esters when treated as above did not yield any diagnostically reliable fragmentation ions which could be used to identify the position of the original double bond. The derivatives of the fatty acid methyl esters containing more than one boronate group produced a rearrangement ion containing two boron atoms which was of relatively high abundance but of little diagnostic value.



The origin of the tropylium ion (m/e 91) in the mass spectra of the benzeneboronate and p -substituted benzeneboronates of 1,2-ethanediol has been fully studied^{220–222}. It is known to arise by two independent pathways involving rearrangement under electron-impact conditions. Cyclic hydrocarbon ions containing 7,8,9 and/or 10 carbon atoms are formed in the electron-impact mass spectra of diols with 1,3-, 1,4-etc. substituents²²³.



$X = H, CH_3, Cl, CH_3O, Br$

Information concerning the GC of the boronate derivatives of diols and ketols is summarized in Table 15.

10.2.2. Carbohydrates. The boronic acids have been used for several years as a protecting group for *cis*-diols in the synthesis and modification of carbohydrates. This has been reviewed by Ferrier²²⁹. For synthetic work, benzeneboronates have been preferred over alkaneboronates as they generally give crystalline derivatives with sharp melting points. An acetone solution of benzeneboronic acid in the presence of an acid catalyst gives a moderately high yield of a mixed acetonide-boronate deriva-

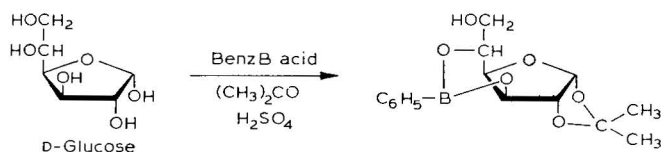
TABLE 15

BORONATE DERIVATIVES USED IN THE GC OF DIOLS AND KETOLS

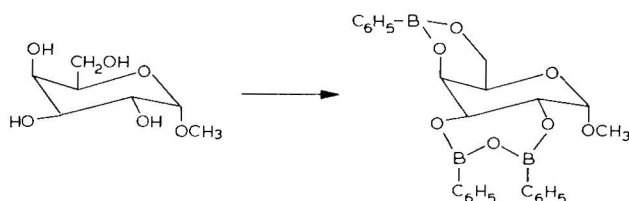
<i>Compound</i>	<i>Boronate derivative</i>	<i>Comments</i>	<i>Ref.</i>
Benzoin	BuB	I, EI-MS	211, 224
Colupulone	BuB	GC, EI-MS	217, 218
Cohumulone	BuB	GC, MS	217, 218
Cyclohexane- <i>cis</i> -1,2-diol	BuB, BenzB	MU, EI-MS	209
Cycloheximide	BuB	I, EI-MS	211, 224
Cytenamide-10,11-dihydrodiol	BuB	GC, EI-MS	214
10,11-Dihydro-10,11-dihydroxy-5H-dibenzo[<i>a,d</i>]cycloheptene	BuB	GC, EI-MS	213
2,5-Dichloro-2',3'-biphenyldiol	BuB	EI-MS	225
2,5-Dichloro-3',4'-biphenyldiol	BuB	EI-MS	225
2,3-Dimethyl-1,2-butanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Dimethyl-2,3-butanediol	BenzB	GC, EI-MS, CI-MS	219
2,6-Dimethyl-1,2-heptanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Dimethyl-2,3-heptanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Dimethyl-2,3-pentanediol	BenzB	GC, EI-MS, CI-MS	219
4,4-Dimethyl-2,3-pentanediol	BenzB	GC, EI-MS, CI-MS	219
Drimane-7 ξ ,8 ξ ,11-triol (11 acetate)	BuB, BenzB	MU, EI-MS	209
4,8,13-Duvatriene-1,3-diol	BuB	GC	210
1,2-Ethanediol	BuB	GC	226
2-Ethyl-1,2-hexanediol	BenzB	GC, EI-MS, CI-MS	219
1,2-Heptanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Heptanediol	BenzB	GC, EI-MS, CI-MS	219
3,4-Heptanediol	BenzB	GC, EI-MS, CI-MS	219
1,2-Hexanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Hexanediol	BenzB	GC, EI-MS, CI-MS	219
3,4-Hexanediol	BenzB	GC, EI-MS, CI-MS	219
2-Hydroxycyclohexanone	BuB	I, EI-MS	211, 224
O-(1-Hydroxyethyl) cyclohexanol	BuB	I, EI-MS	211, 224
O-Hydroxymethylphenol	BuB	MU, EI-MS	209, 211
Humulone	BuB	GC, EI-MS	217, 218
Iminostilbene-10,11-dihydrodiol	BuB	GC, EI-MS	214
Indane- <i>cis</i> -1,2-diol	BuB, BenzB	MU, EI-MS	209
Lupulone	BuB	GC, EI-MS	217, 218
Menthoglycol	BuB	GC, EI-MS	227
2-Methyl-1,2-butanediol	BenzB	GC, EI-MS, CI-MS	219
2-Methyl-2,3-butanediol	BenzB	GC, EI-MS, CI-MS	219
2-Methyl-2,3-heptanediol	BenzB	GC, EI-MS, CI-MS	219
2-Methyl-3,4-hexanediol	BenzB	GC, EI-MS, CI-MS	219
2-Methyl-2,3-pentanediol	BenzB	GC, EI-MS, CI-MS	219
Methyl- <i>erythro</i> -9,10-dihydroxystearate (and <i>threo</i> isomer)	BuB	I, EI-MS	211
Neomenthoglycol	BuB	GC, EI-MS	227
1,2-Nonanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Nonanediol	BenzB	GC, EI-MS, CI-MS	219
3,4-Nonanediol	BenzB	GC, EI-MS, CI-MS	219
4,5-Nonanediol	BenzB	GC, EI-MS, CI-MS	219
1,2-Octanediol	BenzB	GC, EI-MS, CI-MS	219
1,3-Octanediol	MeB, BuB	GC, EI-MS	228
2,3-Octanediol	BenzB	GC, EI-MS, CI-MS	219
3,4-Octanediol	BenzB	GC, EI-MS, CI-MS	219
4,5-Octanediol	BenzB	GC, EI-MS, CI-MS	219

TABLE 15 (continued)

Compound	Boronate derivative	Comments	Ref.
1,2-Pentanediol	BenzB	GC, EI-MS, CI-MS	219
2,3-Pentanediol	BenzB	GC, EI-MS, CI-MS	219
Phenylethyleneglycol	BuB, BenzB	GC, EI-MS	209
	BuB	GC, EI-MS	216
1,3-Propanediol	BuB	GC	226
Pyridoxine	BuB	I, EI-MS	224
2,4,4,-Trimethyl-1,2-pentanediol	BenzB	GC, EI-MS, CI-MS	219



tive with monosaccharides²³⁰. The boronic acids are generally considered to be specific for *cis*-diol groups in carbohydrates. However, the *trans*-1,2-diol group in methyl α -D-galactopyranoside (also methyl α -D-glucopyranoside) reacts with an excess of benzeneboronic acid in a mixture of dimethylformamide and 2,2-dimethoxypropane (room temperature, 30 min) to form a derivative containing a seven-membered di-benzenepyroboronate ring²³¹. The synthesis and characterization by spectroscopy (NMR, EI-MS, OR) was used to confirm the constitution of the benzeneboronate and butaneboronate derivatives of arabinose and xylose²³² and fucose, glucose, fructose and glyceraldehyde²³³ which are derivatives used for GC.



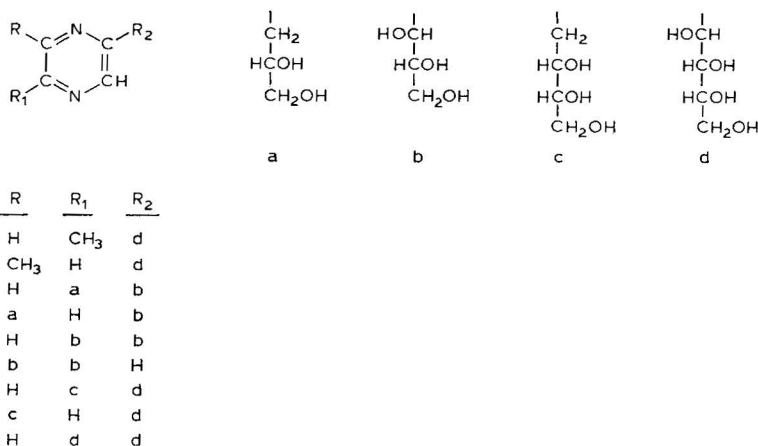
Most of the information concerning the scope of boronic acids to stabilize carbohydrates towards GC has been summarized by Wood and co-workers^{234,235} and by Eisenberg^{236,237}. The boronic acids are particularly suited to the analysis of monosaccharides with even numbers of substituents in the correct spatial configuration for reaction and to hexoses, hexosamines and uronic acids which can be reduced to alditols, aldosaaminitols and aldonoalactones respectively. Monosaccharides containing only *trans* orientated hydroxyl groups (*e.g.*, scyllitol, methyl glucoside, sucrose etc.) do not produce peaks on GC²³⁷. Pyridine is the recommended solvent for reaction and heating is only required if the sugar is insoluble in the solvent. An excess of boronic acid is required to force the reaction to completion²³⁵. Excess butaneboronic acid (if the derivatives contain no free polar groups) does not present a chromatographic problem as the anhydride formed on injection into the GC elutes with the solvent front under the conditions used for the separation of carbohydrates.

Boronate derivatives of carbohydrates with unprotected functional groups such as hydroxyl, amino and phosphate have poor peak shape and require further derivatization prior to analysis. Phosphate groups are protected by methyl ester formation²³⁸ and hydroxyl and amino groups by trimethylsilylation^{234,235,239} or acylation²⁴⁰. No satisfactory peaks were observed on GC for the butaneboronate or butaneboronate TMS ether derivatives of D-galactouronic acid, D-glucuronic acid, D-glucurono-6,3-lactone, 2-acetamido-2-deoxy-D-galactose and 2-acetamido-2-deoxy-D-glucose²³⁵. The butaneboronates of alditols produced single peaks on GC but arabinitol and xylitol had poor peak shape^{235,237}. The formation of TMS ethers with HMDS-TMCS improved the peak shape, but under these conditions, all the alditols (except xylitol) showed the presence of more than one product on GC. The reaction conditions for the formation of the butaneboronate derivatives of fucose, arabinose, xylose and butaneboronate-trimethylsilyl derivatives of fructose, galactose, mannose and glucose have been studied²³⁵. The boronate-TMS ether derivatives with the exception of the derivative of mannose are stable for at least one week when stored in the silylation medium. A mixture of BSTFA-TMCS (1:1) was used for the formation of the TMS ethers of the methaneboronate and butaneboronate derivatives of rhamnopyranose, flucopyranose, mannopyranose, glucopyranose, galactopyranose, 2-acetamido-2-deoxy-D-glucopyranose, 2-acetamido-2-deoxy-D-galactopyranose and 2-acetamido-2-deoxy-D-glucopyranose²³⁹. Acylation of the free hydroxyl groups of the butaneboronate derivatives of glucose, galactose, mannose, fructose and sorbose was complete in one hour at room temperature in a mixture of pyridine and acetic anhydride²⁴⁰. The boronate acetate derivatives had good peak shape on GC.

Under carefully controlled conditions, quantitative (or reproducible) yields of cyclic boronates of simple sugars can be obtained and these derivatives are useful in quantitative analysis. The butaneboronate derivatives of glucose and fructose were used for their determination in honey²⁴¹, the butaneboronate of 1,2:5,6-dianhydrogalactitol for its determination in plasma²⁴², the tris(butaneboronate) of sorbitol for its determination in pharmaceutical preparations^{243,244} and iduronic and glucuronic acid were determined after conversion to aldonic acids as their tris(butaneboronate) derivatives²⁴⁵.

The mass spectra of the boronate-TMS carbohydrate derivatives enable the number of carbon atoms (pentose or hexose), the ring size (furanose and pyranose) and the stereochemistry of the hydroxyl groups to be determined²³⁹. The number of boronate groups identified in the derivative indicates the number of hydroxyl groups in the correct spatial configuration for boronate formation and the number of TMS groups indicates how many further hydroxyl groups are present. In mixed derivatives the boronate group does not strongly direct the mode of fragmentation and this is usually dominated by the TMS²³⁹, acetate²⁴⁰ or phosphate²³⁸ substituent. The mass spectra of carbohydrate benzeneboronates have been reviewed²²⁹. Benzeneboronate-TMS ether derivatives of nucleosides have been used for the determination of the base sequence in polynucleosides by mass spectrometry without chromatographic separation^{246,247}.

The reaction of sugars with ammonia in weakly acid medium results in the formation of a complex mixture of polyhydroxyalkylpyrazines which can be separated by GC of their butaneboronate-TMS ethers and identified by MS²⁴⁸. The structures of the nine polyhydroxyalkylpyrazines investigated are given below:



The butaneboronate-TMS derivatives were generally more volatile than the completely trimethylsilylated derivatives on GC but unlike the latter showed some minor peaks in addition to the main peak in some cases.

Those carbohydrates which have been separated by GC of their boronate derivatives are summarized in Table 16.

10.2.3. Steroids. The C-17 side chain of the corticosteroids is thermally labile and eliminated at the temperatures employed for their separation by GC unless protected by the formation of a suitable derivative. The boronic acids have been thoroughly evaluated for this purpose^{46,224,250-254}. Methaneboronic acid, butaneboronic acid, *tert.*-butaneboronic acid, cyclohexaneboronic acid and benzeneboronic acid have all been used as derivatizing reagents. The cyclohexaneboronates and benzeneboronates had inconveniently long retention times. The methaneboronates are very volatile with retention times generally less than those of the TMS derivatives. The *tert.*-butaneboronates also have surprisingly short retention times but are very air sensitive and must be handled under nitrogen. The *tert.*-butaneboronic acid reagent was prepared in the hope that the bulky *tert.*-butyl group would enable derivatives to be prepared which were more hydrolytically stable. This was not found to be the case. The butaneboronate derivatives provide a convenient compromise between volatility and hydrolytic stability and have been widely used. Retention index values for a number of corticosteroid boronate derivatives are compiled in Table 17.

Reaction of the corticosteroid side chain with a boronic acid is rapid at room temperature. For 17 α ,20-diols, 20,21-diols and 17 α ,20,21-triols, reaction is complete within 5 min when 1 mole equivalent of boronic acid is used²⁵². In the case of the 17 α , 20,21-triols, reaction most probably occurs at the C-17,21 hydroxyl groups²²⁴. For derivatization of the 17,21-dihydroxy-20-ketone group a slight excess (1.1 mole equivalents) of boronic acid was required and a reaction time of 15 min for complete reaction. If there are no further free hydroxyl groups in the steroid then a larger excess of boronic acid can be used. Direct injection into the gas chromatograph of a corticosteroid with free hydroxyl groups at C-3, 11 or 20 results in excessive peak tailing and a reduction in peak height for the derivative unless the free hydroxyl group is also protected as its TMS or acetate derivative. Reaction of the C-20,21 ketols even

TABLE 16

CARBOHYDRATES FORMING BORONATE DERIVATIVES USED IN GAS CHROMATOGRAPHY

<i>Compound</i>	<i>Boronic acid</i>	<i>Comments</i>	<i>Ref.</i>
2-Acetamido-2-deoxy-D-galactopyranose	MeB, BuB	GC, EI-MS	239
2-Acetamido-2-deoxy-D-glucopyranose	MeB, BuB	GC, EI-MS	239
2-Acetamido-2-deoxy-D-gulopyranose	MeB, BuB	GC, EI-MS	239
Allitol	BuB	GC	237
Allose	BuB	GC, EI-MS	240
Altose	BuB	GC, EI-MS	240
Arabinitol	BuB	GC	235
Arabinose	BuB	GC	235
	BuB, BenzB	GC	232
	BuB, BenzB	GC, EI-MS	239
	BuB	GC	234
	BuB	GC	237
Ascorbic acid	BuB	GC	237
Chiroinositol	BuB	GC	237
2-Deoxy-D-erythro-pentose	BuB	GC	235
1,2:5,6-Dianhydrogalactitol	BuB	GC, EI-MS	242
Erythritol	BuB	GC	234
Fructose	BuB	GC	234
	BuB	GC, EI-MS	235
	BuB	GC, EI-MS	240
	BuB, BenzB	GC, EI-MS	233
Fructose 1-phosphate	BuB, MeB	GC	237
	BuB, MeB	GC, EI-MS	238
Fructose 6-phosphate	BuB, MeB	GC	238
Fucitol	BuB	GC	237
	BuB	GC	234
Fucopyranose	MeB, BuB	GC, EI-MS	239
Fucose	BuB	GC	249
	BuB	GC	237
	BuB, BenzB	GC, EI-MS	233
	BuB	GC	236
	BuB	GC	234
Galactitol	BuB	GC	235
	BuB	GC	237
	BuB	GC	234
Galactopyranose	MeB, BuB	GC, EI-MS	239
Galactose	BuB	GC	234
	MeB, BuB	GC, EI-MS	240
	BuB	GC, EI-MS	235
	BuB	GC, EI-MS	237
Galactose 1-phosphate	MeB, BuB	GC, EI-MS	238
Galactose 6-phosphate	MeB, BuB	GC	237
Galactoseaminitol	BuB	GC	237
Gluconolactone	BuB	GC	237
Glucopyranose	MeB, BuB	GC, EI-MS	239
Glucitol	BuB	GC	237
	BuB	GC	235
	BuB	GC	234
	MeB, BuB	GC	234
Glucuronic acid 6-phosphate	MeB, BuB	GC, EI-MS	245

TABLE 16 (continued)

<i>Compound</i>	<i>Boronic acid</i>	<i>Comments</i>	<i>Ref.</i>
Glucose	BuB	GC	234
	MeB, BuB	GC, EI-MS	240
	BuB, BenzB	GC, EI-MS	233
	BuB, BenzB	GC	237
	BuB, BenzB	GC, EI-MS	237
Glucuronic acid	BuB, BenzB	GC, EI-MS	245
Glucose 6-phosphate	BuB	GC, EI-MS	235
Glucose aminitol	BuB	GC	235
Glucuronic acid	BuB	GC	245
Glyceraldehyde	BuB, BenzB	GC, EI-MS	233
Gulolactone	BuB	GC	237
Iditol	BuB	GC	237
Idonolactone	BuB	GC	237
Idose	BuB	GC, EI-MS	240
Iduronic acid	BuB	GC	245
Lyxose	BuB	GC	234
Methyl-D-Mannopyranose	MeB, BuB, BenzB	GC, EI-MS	239
Mannitol	BuB	GC	234
	BuB	GC	244
	BuB	GC	245
Sorbitol	BuB	GC	243
	BuB	GC	244
Sorbose	BuB	GC	240
Tagatose	BuB	GC	240
Talose	BuB	GC	240
Xylitol	BuB	GC	234
	BuB	GC	235
	BuB	GC	237
Xylose	BuB	GC	234
	BuB, BenzB	GC	249
	BuB, BenzB	GC, EI-MS	232
	BuB	GC	235
	BuB	GC	237

with excess boronic acid does not proceed to completion and there is evidence that partial decomposition of the derivative may also occur on GC. The boronate derivatives of 17,20- and 20,21-diols and 17,20,21-triols are stable to both BSA and HMDS-TMCS when used to form TMS derivatives of unprotected hydroxyl groups and to acetic anhydride-pyridine used to form acetate derivatives^{46,224,252}. Under similar conditions the 17,21-dihydroxy-20-ketone and 20, 21-ketal boronates gave a mixture of products with loss of the boronate group. Methyloxime derivatives of ketones can be formed prior to or after boronate formation without problems. The boronate derivatives of the 17,20- and 20,21-diols and 17,20,21-triols yielded stable derivatives which resisted hydrolysis and could be submitted to TLC. The boronate ester derivative can be displaced from the corticosteroid side chain by addition of an excess of 1,3-propanediol for recovery of the original steroid^{224,250-252}.

An interesting cyclic derivative of 18-hydroxy-11-deoxycorticosterone is formed by reaction with methanaboronic acid involving a molecular re-arrangement in the corticosteroid side chain²⁵⁴.

TABLE 17

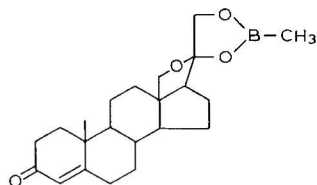
RETENTION INDEX VALUES OR METHYLENE UNITS FOR CORTICOSTEROID BORON-ATES OBTAINED BY GC ON OV-17

6 ft. \times 4 mm I.D. glass column of 1% OV-17 on Gas-Chrom Q, 100–120 mesh. MU values determined by temperature programming from 230° at 2° min.

<i>Corticosteroid</i>	<i>Boronic acid</i>	<i>Retention index for derivative</i>	<i>Column temperature (°C)</i>
5 β -Pregnane-3 α ,17 α ,20 α -triol	MeB	3010	240
	t-BuB	3050	240
	BuB	3265	MU
	CHB	3590	250
	BenzB	3775	250
5 β -Pregnane-3 α ,17 α ,20 β -triol	MeB	2970	240
	t-BuB	3010	240
	BuB	3265	MU
	BenzB	3775	250
5 β -Pregnane-3 α ,11 β ,17 α ,20 β -tetrol	MeB	3255	250
	t-BuB	3270	250
	BuB	3480	MU
20 β ,21-Dihydroxypregn-4-ene-3-one	MeB	3380	250
	t-BuB	3520	250
	BuB	3680	MU
	CHB	4030	250
	BenzB	4330	250
5 α -Pregnane-3 α ,11 β ,20 α ,21-tetrol	MeB	3460	250
	t-BuB	3600	250
5 α -Pregnane-3 α ,11 β ,20 β ,21-tetrol	MeB	3470	250
	t-BuB	3565	240
17 α ,20 α ,21-Pregnan-4-ene-3-one	MeB	3595	250
	t-BuB	3650	250
	CHB	4205	250
17 α ,20 β ,21-Trihydroxy-pregnan-4-ene-3-one	MeB	3620	250
	t-BuB	3650	250
	BuB	3835	250
	CHB	4080	250
	BenzB	4345	250
5 α -Pregnane-3 β ,11 β ,17 α ,20 β ,21-pentol	MeB	3650	250
	t-BuB	3715	250
3 α ,17 α ,20 α ,21-Tetrahydroxy-5 β -pregnane-11-one	MeB	3490	250
	t-BuB	3570	250
	BuB	3800	MU
17 α ,21-Dihydroxypregnan-4-ene-3,20-dione	MeB	3360	250
	t-BuB	3400	250
	BuB	3580	MU
	CHB	3965	250
	BenzB	4120	250
17 α ,21-Dihydroxy-5 β -pregnane-3,20-dione	MeB	3175	240
	t-BuB	3215	240
	BuB	3400	MU
	CHB	3710	250
	BenzB	3885	250
17 α ,21-Dihydroxypregnan-4-ene-3,11,20-trione	MeB	3450	250
3 α ,17 α ,21-Trihydroxy-5 β -pregnane-20-one	MeB	3180	250
	t-BuB	3220	250
	BuB	3345	MU

TABLE 17 (continued)

Corticosteroid	Boronic acid	Retention index for derivative	Column temperature (°C)
	t-BuB	3490	250
	BuB	3660	MU
	CHB	4065	250
	BenzB	4230	250
17 α ,21-Dihydroxy-5 β -pregnane-3,11,20-trione	MeB	3300	250
	t-BuB	3305	250
	BuB	3660	MU
	CHB	3825	250
	BenzB	3990	250
3 α ,17 α ,21-Trihydroxy-5 β -pregnane-11,20-dione	MeB	3270	250
	t-BuB	3270	240
	BuB	3465	MU
11 β ,17 α ,21-Trihydroxypregnan-4-ene-3,20-dione	MeB	3630	250
	t-BuB	3660	250
	BuB	3890	250
3 α ,11 β ,17 α ,21-Tetrahydroxy-5 β -pregnane-20-one	MeB	3360	240
	t-BuB	3400	240
	BuB	3605	MU
21-Hydroxypregnan-4-ene-3,20-dione	MeB	3310	250
	t-BuB	3480	250
21-Hydroxy-5 β -pregnane-3,20-dione	MeB	3220	250
	t-BuB	3380	250
3 β -21-Dihydroxy-pregnane-5-ene-20-one	MeB	3150	250
	t-BuB	3325	MU
	BuB	3470	250
	BenzB	3850	250



Methaneboronate of 18-hydroxy-11-deoxycorticosterone

The mass spectra of the corticosteroid boronates are characterized by a prominent molecular ion fragmenting to produce abundant daughter ions characteristic of the parent steroid²⁵³. In most cases the positive charge is localized on the steroid nucleus yielding diagnostically informative mass spectra. The chemical-ionization mass spectra of the methaneboronate derivative of 18-hydroxy-11-deoxycorticosteroid has the quasi $[M+1]^+$ molecular ion as its base peak with very little further fragmentation²⁵⁴.

Steroids which have been separated by GC as their boronate derivatives are summarized in Table 18.

10.2.4. Prostaglandins. The ability to form a boronate derivative has been used to distinguish the prostaglandin F series from the E series. Only the F prostaglandins have a 9 α ,11 α *cis*-diol group, which is the site of boronate formation. The boronic acids react specifically with the *cis*-diol group as the prostaglandin F $_{\beta}$ series

TABLE 18

BORONATE DERIVATIVES FOR THE GAS CHROMATOGRAPHIC SEPARATION OF STEROIDS

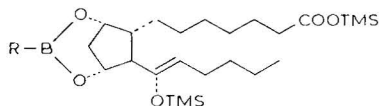
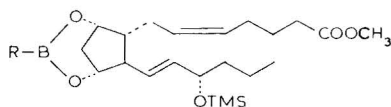
<i>Steroid</i>	<i>Boronate derivative</i>	<i>Comments</i>	<i>Ref.</i>
<i>Androstanes</i>			
3 β ,16 α ,17 α -Trihydroxyandrost-5-ene	BuB	MU, EI-MS	250
3 β ,16 β ,17 β -Trihydroxyandrost-5-ene	BuB	MU, EI-MS	250
<i>Cholestanes</i>			
2 β ,3 β -Dihydroxy-5 α -cholestane	BuB	GC	255
<i>Pregnanes</i>			
21-Hydroxy-5 β -pregnane-3,20-dione	BuB	MU, EI-MS	252
21-Hydroxy-5 α -pregnane-3,20-dione	MeB, t-BuB	I, EI-MS	252
21-Hydroxypregnan-4-ene-3,20-dione	MeB, t-BuB	I, EI-MS	252
18-Hydroxy-11-deoxycorticosteroid	MeB	I, EI-MS, CI-MS	254
3 β ,21-Dihydroxypregnan-5-ene-20-one	MeB, BuB, t-BuB		252
	BenzB	I, EI-MS	
	BuB	MU, EI-MS	250
17 α ,21-Dihydroxy-5 β -pregnane-3,20-dione	MeB, BuB, t-BuB		
	CHB, BenzB	I, EI-MS	250
	BuB	MU, EI-MS	250
17 α ,21-Dihydroxypregnan-4-ene-3,20-dione	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB		252
	CHB, BenzB	I, EI-MS	
	MeB	I, EI-MS	46
17 α ,21-Dihydroxy-5 β -pregnane-3,11,20-trione	MeB, BuB, t-BuB		
	CHB, BenzB	I, EI-MS	252
	BuB	MU, EI-MS	250
17 α ,21-Dihydroxypregnan-4-ene-3,11,20-trione	MeB, BuB, t-BuB		
	CHB, BenzB	I, EI-MS	252
	MeB, BuB		
	t-BuB, BenzB	I, EI-MS	251
	BuB	MU, EI-MS	250
20 β ,21-Dihydroxypregn-4-ene-3-one	MeB, BuB, t-BuB		
	CHB, BenzB	I, EI-MS	252
	MeB	I, EI-MS	46
3 α ,17 α ,20 α -Trihydroxy-5 β -pregnane	BuB, BenzB	MU, EI-MS	209
	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB		
	CHB, BenzB	MU, EI-MS	251
-3-TMS-ether	BuB	MU, EI-MS	250
-3-acetate ester	BuB	MU, EI-MS	250
3 α ,17 α ,20 β -Trihydroxy-5 β -pregnane	MeB	I, EI-MS	46
	MeB, BuB, t-BuB		
	CHB, BenzB	I, EI-MS	251
3 β ,17 α ,20 α -Trihydroxypregn-5-ene	BuB	MU, EI-MS	250
3 β ,17 α ,20 β -Trihydroxypregn-5-ene	BuB	MU, EI-MS	250
3 α ,17 α ,21-Trihydroxy-5 β -pregnane-3-one	MeB, BuB, t-BuB	MU, EI-MS	252
3 α ,20 α ,21-Trihydroxy-5 β -pregnane-11-one	BuB	MU, EI-MS	250
3 α ,17 α ,21-Trihydroxy-5 β -pregnane-20-one	BuB	MU, EI-MS	250
	BuB	MU, EI-MS	252
	BuB	GC, EI-MS	224
3 α ,17 α ,21-Trihydroxypregnane-11,20-dione	BuB	MU, EI-MS	252
	MeB, BuB, t-BuB	I, EI-MS	250
17 α ,20 α ,21-Trihydroxypregn-4-ene-3-one	BuB	GC, EI-MS	224

TABLE 18 (continued)

Steroid	Boronate derivative	Comments	Ref.
17 α ,20 β ,21-Trihydroxypregn-4-ene-3-one	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB		252
	CHB	I, EI-MS	252
	MeB	I, EI-MS	46
	MeB, BuB, t-BuB		
11 β ,17 α ,21-Trihydroxypregn-4-ene-3,20-dione	CHB, BenzB	I, EI-MS	252
	BuB	GC, EI-MS	224
	BuB	MU, EI-MS	250
3 α ,11 β ,17 α ,20 β -Tetrahydroxy-5 β -pregnane	MeB, BuB, t-BuB	I, EI-MS	252
	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB	I, EI-MS	252
3 α ,11 β ,20 α ,21-Tetrahydroxy-5 α -pregnane	MeB, t-BuB	MU, EI-MS	252
3 α ,11 β ,20 α ,21-Tetrahydroxy-5 α -pregnane	t-BuB	MU, EI-MS	250
3 α ,11 β ,20 β ,21-Tetrahydroxy-5 α -pregnane	MeB, t-BuB	MU, EI-MS	250
3 α ,11 β ,20 β ,21-Tetrahydroxy-5 β -pregnane	t-BuB	MU, EI-MS	250
3 α ,11 β ,17 α ,21-Tetrahydroxy-5 β -pregnane-20-one	BuB	MU, EI-MS	250
3 α ,17 α ,20 α ,21-Tetrahydroxy-5 β -pregnane-11-one	MeB, BuB, t-BuB	MU, EI-MS	252
	BuB	MU, EI-MS	250
	MeB, BuB, t-BuB	I, EI-MS	252
3 α ,17 α ,20 β ,21-Tetrahydroxy-5 β -pregnane-11-one	BuB	MU, EI-MS	250
3 β ,11 β ,17 α ,20 β ,21-Pentahydroxy-5 β -pregnane	MeB, t-BuB	MU, EI-MS	252

which contain a *trans*-diol group do not form a derivative²⁵⁶. The selectivity of the reaction for the *cis*-diol group has been important in identifying the presence of the prostaglandin F $_{\alpha}$ series in biological samples (sheep blood²⁵⁷, human semen²⁵⁸, human aortal media²⁵⁹, rat stomach homogenate²⁶⁰, and rat uterus media²⁶¹) when only small quantities of extracts were available for study.

The prostaglandins are multifunctional compounds and mixed derivatives are prepared for their GC analysis. Either the cyclic boronate TMS ether TMS ester derivative²⁵⁶ or the cyclic boronate TMS ether methyl ester derivative were prepared²⁵⁷⁻²⁶⁵. A disadvantage of the cyclic boronate TMS ether TMS ester derivatives was their ease of solvolysis. The butaneboronate derivative of prostaglandin F $_{1\alpha}$ is slowly converted to the fully trimethylsilylated derivative beginning after about 2 h at room temperature in a solution of Trisil Z²⁵⁶. In a 5% solution of BSTFA in hexane, the butaneboronate TMS ether methyl ester derivative of prostaglandin F $_{2\alpha}$ was stable

Prostaglandin F $_{1\alpha}$ cyclic boronate TMS ether TMS ester derivativeProstaglandin F $_{2\alpha}$ cyclic boronate TMS ether methyl ester derivative

at room temperature for up to ten days and considerably longer at -15° (ref. 263). Brief mention has been made of the formation of a 19-hydroxyprostaglandin F_{1a} and F_{2a} butaneboronate *tert.*-butyldimethylsilyl ether methyl ester derivative which have as their base peak, the ion $[M-\text{tert.-Bu}]^{+}$ in the mass spectrum. Various boronic acids have been evaluated for the separation of prostaglandin F_{1a} , F_{2a} and F_{3a} as their cyclic boronate TMS ether methyl ester derivatives (Table 19)²⁶⁵. The prostaglandin F_{2a} and F_{3a} derivatives are not separated adequately on packed or capillary columns of OV-1 or OV-17. Chemical-ionization MS can be used to distinguish between the different F_a prostaglandins by identification of the base peak $[M-RBO_2H_2-TMSO]^{+}$ in the mass spectrum which occurs at a different m/e value for each prostaglandin²⁶⁵. In the electron-impact mass spectra the base peak $[M-71]^{+}$ of the prostaglandin F_{2a} derivative occurs at the same m/e value (393 a.m.u.) as the base peak of the prostaglandin F_{3a} $[M-69]^{+}$ derivative. This ion is often chosen for the determination of prostaglandin F_{2a} by single ion monitoring^{259,263,264}. As the prostaglandin F_{2a} and F_{3a} derivatives are not separated chromatographically some problems may arise in confirming the identities of these two prostaglandins.

TABLE 19

RETENTION INDEX VALUES FOR THE CYCLIC BORONATE TMS ETHER METHYL ESTER DERIVATIVES OF PROSTAGLANDINS F_{1a} , F_{2a} AND F_{3a} ON OV-1

2 m \times 4 mm I.D. glass column of 1% OV-1 on Gas-Chrom Q (100–120 mesh) at 225° isothermally with a nitrogen flow-rate of 50 ml min⁻¹.

Boronate derivative	Prostaglandin	Retention index
Methaneboronate	F_{1a}	2625
	F_{2a}	2585
	F_{3a}	2590
Butaneboronate	F_{1a}	2885
	F_{2a}	2850
	F_{3a}	2845
Cyclohexaneboronate	F_{1a}	3160
	F_{2a}	3125
	F_{3a}	3120
Benzeneboronate	F_{1a}	3230
	F_{2a}	3185
	F_{3a}	3180

The prostaglandin F_a series which have been separated by GC of their boronate derivatives are summarized in Table 20.

10.2.5. Lipids. Sphingosines, ceramides, monoglycerides and monoalkyl glyceryl ethers can be determined by GC after formation of their cyclic boronate derivatives^{254,266–269}. The sphingosines are sphingolipid long-chain bases (sphinganine, *D-erythro*-1,3-dihydroxy-2-amino-octadecane; 4-sphingenine, *D-erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene; 4-*D*-hydroxysphinganine, *D-ribo*-1,3,4-trihydroxy-2-amino-octadecane) and are readily converted to their boronate esters by reaction with a slight excess of boronic acid (1.1 mole equivalent) in pyridine at room temperature for 10 min^{254,266}. MS indicates that boronate formation takes place at the 1,3-diol group of the 2-amino-1,3-diol side chain and that in the case of 4-*D*-hydroxy-

TABLE 20

BORONATE DERIVATIVES FOR THE GAS CHROMATOGRAPHIC ANALYSIS OF PROSTAGLANDINS

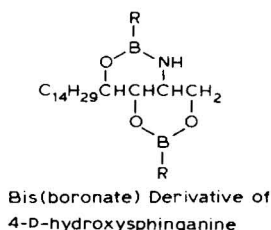
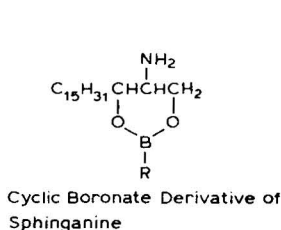
<i>Prostaglandin</i>	<i>Boronate derivative</i>	<i>Comments</i>	<i>Ref.</i>
Prostaglandin F _{1α}	BuB	GC, EI-MS	256
	MeB, BuB, CHB		
	BenzB	I, EI-MS, CI-MS	265
	BuB	GC, EI-MS	262
	BuB	GC, EI-MS	263
	BuB	GC, EI-MS	264
Prostaglandin F _{2α}	BuB	GC, EI-MS	256
	MeB, BuB, CHB		
	BenzB	I, EI-MS, CI-MS	265
	MeB, BuB, CHB		
	BenzB	I, EI-MS	259
	BuB	GC, EI-MS	263
	BuB	GC, EI-MS	257
	MeB, BuB, CHB		
	BenzB	I, EI-MS, CI-MS	265
Prostaglandin F _{3α}	BuB	GC, EI-MS	258
19-Hydroxyprostaglandin F _{1α}	BuB	GC, EI-MS	258
19-Hydroxyprostaglandin F _{2α}	BuB	GC, EI-MS	261
6-Ketoprostaglandin F _{1α}	BuB	GC, EI-MS	260

TABLE 21

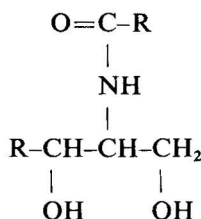
RETENTION INDICES FOR SPHINGOSINE BORONATE DERIVATIVES ON GAS CHROMATOGRAPHY

2 m × 3 mm I.D. glass column of 1% stationary phase on Gas-Chrom Q (100–120 mesh). Flow-rate 50 ml min⁻¹. DMAM = N,N-dimethylaminomethylene.

<i>Compound</i>	<i>Derivative</i>	<i>Column temperature (°C)</i>	<i>Stationary phase</i>	
			<i>OV-1</i>	<i>OV-17</i>
Sphinganine	MeB	200	2315	2480
	BuB	230	2590	2740
	BenzB	230	2940	3220
	MeB, DMAM	250	2570	2765
	BuB, DMAM	230	2840	3020
	BenzB, DMAM	270	3260	3535
	N-Acetyl, MeB	230	2600	2835
	N-Acetyl, BuB	270	2850	3080
	N-Acetyl, BenzB	270	3210	3595
	Acetone Schiff base, MeB	230	2420	2565
	Acetone Schiff base, BuB	230	2675	2815
	Acetone Schiff base, BenzB	270	3040	3310
4-Sphingenine	MeB	200	2305	2485
	BenzB	230	2940	3270
	Acetone Schiff base, MeB	230	2420	2580
	Acetone Schiff base, BenzB	270	3030	3360
4 D-Hydroxy-sphinganine	Bis-MeB	230	2425	2570



sphinganine a bis(boronate) derivative was formed. The sphinganine boronate and the bis(boronate) derivative of 4-D-hydroxysphinganine had poor chromatographic properties unless the amine group was also derivatized. The boronate derivative was stable to the subsequent conversion of the amine group to an N-acetyl, N,N-dimethylaminomethylene or Schiff base derivative. The Schiff base derivatives had the shortest retention times and superior peak shape on GC. Retention index data for some derivatives of sphingosine are given in Table 21. All derivatives with the exception of the N,N-dimethylaminomethylene derivatives could be stored for several days at room temperature without decomposition and even longer in a refrigerator^{254,266,269}.



General Structure of Ceramides

Ceramides are N-acyl sphingosines containing a 2-amido-1,3-diol structure. They form cyclic boronate derivatives via the 1,3-diol group. The methaneboronate derivatives were formed by adding a 1.1 molar excess of boronic acid (2.2 molar in the case of ceramides derived from 2-hydroxyacids) to the ceramide in pyridine and allowing the reaction to proceed for 10 min at room temperature²⁶⁷. For ceramides derived from the enzymatic hydrolysis of lysolecithin and sphingomyelin a 1.5 mole excess of boronic acid was reacted with the ceramide in ethyl acetate solution for 30 min at room temperature²⁶⁸. Retention index data for some ceramide methaneboronate derivatives is given in Table 22. The derivatives were stable in solution for several days at room temperature, could be submitted to TLC without decomposition and were stable to treatment with pyridine-HMDS-TMS (5:4:3) at room temperature for 30 min.

Lipids which have been determined by GC after formation of their boronate derivatives are summarized in Table 23.

10.2.6. Hydroxyamines and hydroxyacids. The butaneboronate derivatives of hydroxyamines and hydroxyacids have good peak shape and in the case of the hydroxyacids, longer retention times by 300–400 retention index units compared to the methyl hydroxyesters on OV-17 (ref. 211). Boronate derivatives can be formed with α and β substituted hydroxyacids and amines at room temperature in an anhydrous

TABLE 22

RETENTION INDICES FOR CERAMIDE AND MONOALKYL GLYCERIDE METHANE-BORONATES ON OV-1 AS STATIONARY PHASE

Compound	Retention index
<i>Ceramides*</i>	
N-Myristoyl sphinganine	3702
N-Palmitoyl sphinganine	3896
N-Palmitoleoyl sphinganine	3886
N-Stearoyl sphinganine	4097
N-Oleoyl sphinganine	4073
N-Linoleoyl sphinganine	4074
N-Palmitoyl 4-sphingenine	3912
N-Palmitoleoyl 4-sphingenine	3898
<i>Monoalkyl glycerides**</i>	
Glyceryl 1-hexadecanoate	2355
Glyceryl 1-hexadecanoate	2373
Glyceryl 1-heptadecanoate	2478
Glyceryl 1-octadecanoate	2552
Glyceryl 1-octadecanoate	2572

* 40 m \times 0.55 mm I.D. capillary column, pretreated with silanox, temperature 300°, helium flow-rate 15 ml min⁻¹.

** 30 m \times 0.5 mm I.D. capillary column, pretreated with silanox, temperature 230°.

solvent. The derivatives are stable to storage for at least 2–3 days in an anhydrous solvent and several derivatives were stable to TLC²¹¹. Retention index values for some representative hydroxyamines and hydroxyacids are summarized in Table 24.

The salicylate group in cannabinolic acids was converted to its boronate derivative by adding an excess of boronic acid to the sample dissolved in pyridine-benzene (1:1). After 30 min at room temperature, the solvent was evaporated under nitrogen and the procedure repeated again²⁷⁰. A double derivatization technique was essential to remove water and force the reaction to completion. The boronate derivatives were stable to treatment with CH₃CN–BSTFA–TMCS (2:2:1) at room temperature for thirty minutes and could be stored for several weeks at 4° without decomposition. The benzenboronate derivatives like the TMS derivatives were not thermally stable with some decomposition occurring on-column. The methaneboronate and butaneboronate derivatives were thermally stable and had good peak shape. The methaneboronate derivatives had comparable retention times to those of the TMS derivatives, whereas the butaneboronates had longer retention times and methylene unit values about three units higher. The butaneboronate derivatives enabled a complete separation of the cannabinoids from their acids to be achieved on a single packed column.

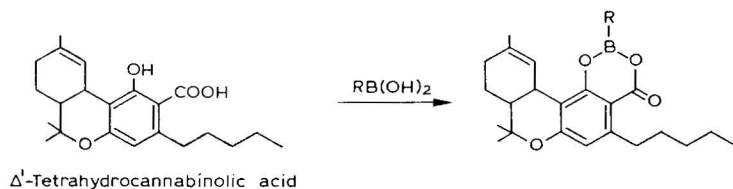


TABLE 23

BORONATE DERIVATIVES USED IN THE GAS CHROMATOGRAPHY OF LIPIDS

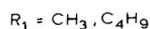
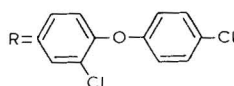
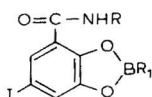
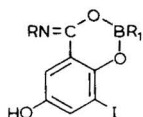
<i>Compound</i>	<i>Boronic acid</i>	<i>Comments</i>	<i>Ref.</i>
<i>Hydroxyfatty acid alkyl esters</i>			
4,5-Dihydroxydocosanoate	BuB, MeB	GC, EI-MS	64
8,9-Dihydroxydodecanoate	BuB, MeB	GC, EI-MS	64
9,10-Dihydroxyoctadecanoate	BuB, MeB	GC, EI-MS	64
5,6,8,9,11,12-Hexahydroxydodecanpropionate	BuB, MeB	GC, EI-MS	64
9,10,12,13,15,16-Hexahydroxyoctadecanpropionate	BuB, MeB	GC, EI-MS	64
4,5,7,8-Tetrahydroxydocosanethanoate	MeB	GC, EI-MS	64
9,10,12,13-Tetrahydroxyoctadecanethanoate	BuB, MeB	GC, EI-MS	64
<i>Glyceryl ethers</i>			
1-Hexadecyl glyceryl ether	MeB	I, EI-MS, CI-MS	254
2-Hexadecyl glyceryl ether	MeB	I, EI-MS, CI-MS	254
<i>Glyceryl alkyl esters</i>			
Glyceryl 1-heptadecanoate	MeB	I, EI-MS	268
Glyceryl 1-myristate	BuB, BenzB	MU, EI-MS	209
Glyceryl 1-octadecenoate	MeB	I, EI-MS	268
Glyceryl 1-palmitate	MeB	I, EI-MS, CI-MS	254
	MeB	I, EI-MS	268
Glyceryl 1-palmitoleate	MeB	I, EI-MS	268
Glyceryl 1-stearate	MeB	I, EI-MS	268
	MeB	I, EI-MS, CI-MS	254
<i>Sphingosines</i>			
Dihydrosphingosine	MeB	I, EI-MS, CI-MS	64
N-Ethyl sphinganine	MeB, BuB, BenzB	GC, EI-MS, CI-MS	228
N-Ethyl 4-sphingenine	MeB, BuB, BenzB	GC, EI-MS, CI-MS	228
4D-Hydroxysphinganine	MeB, BuB, BenzB	GC, EI-MS, CI-MS	228
	MeB, BuB, BenzB	I, EI-MS, CI-MS	266
4-Sphingenine	MeB, BuB, BenzB	I, EI-MS, CI-MS	266
<i>Ceramides</i>			
N-Behenoyl-4-sphingenine	MeB	I, EI-MS	268
N-2-Hydroxystearoyl-4-sphingenine	MeB	I, EI-MS	267
N-Lingnoceronyl-4-sphingenine	MeB	I, EI-MS	268
N-Linoleoyl sphinganine	MeB	I, EI-MS	267
N-Myristoyl sphinganine	MeB	I, EI-MS	267
N-Myristoyl-4-sphingenine	MeB	I, EI-MS	267
N-Nervonoyl-4-sphingenine	MeB	I, EI-MS	268
N-Oleoyl sphinganine	MeB	I, EI-MS	267
N-Palmitoleoyl sphinganine	MeB	I, EI-MS	267
N-Palmitoleoyl-4-sphingenine	MeB	I, EI-MS	267
	MeB	I, EI-MS	267
N-Palmitoyl-D,L-dihydrosphingosine	MeB	I, EI-MS	254
N-Palmitoyl-4-heptadecasphingenine	MeB	I, EI-MS	267
N-Palmitoyl-4-hexadecasphingenine	MeB	I, EI-MS	267
N-Palmitoyl-4-sphingenine	MeB	I, EI-MS	268
	MeB	I, EI-MS	267
N-Stearoyl-D,L-dihydrosphingosine	MeB	I, EI-MS	254
N-Stearoyl-4-dodecasphingenine	MeB	I, EI-MS	268
N-Stearoyl-4-hexadecenylsphingenine	MeB	I, EI-MS	268
N-Stearoyl sphinganine	MeB	I, EI-MS	267
N-Stearoyl-4-sphingenine	MeB	I, EI-MS	268

TABLE 24

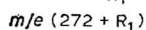
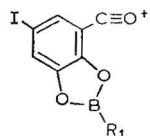
RETENTION INDEX VALUES FOR SOME BUTANEBORONATE DERIVATIVES OF HYDROXYACIDS AND HYDROXYAMINES ON OV-17

Column temperature not given. 6 ft. \times $\frac{1}{8}$ in. I.D. glass column of 1% OV-17 on Gas-Chrom Q.

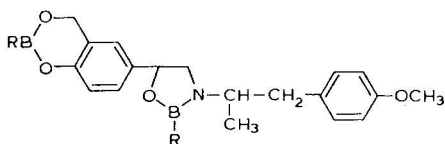
Compound	Retention index
<i>α-Hydroxyacids</i>	
Lactic Acid	1200
α -Hydroxyisobutyric acid	1170
α -Hydroxymyristic acid	2245
Mandelic acid	1805
Tartaric Acid	1940
<i>β-Hydroxyacids</i>	
3-Hydroxypropionic acid	1420
3-Hydroxybutyric acid	1410
<i>cis</i> -2-Hydroxycyclohexanecarboxylic acid	1747
<i>trans</i> -2-Hydroxycyclohexanecarboxylic acid	1768
Salicylic Acid	1792
3-Methoxysalicylic acid	2110
4-Methoxysalicylic acid	2145
6-Methoxysalicylic acid	2180
Thymotic acid	2035
2-Hydroxy-1-naphthoic acid	2450
2-Hydroxy-3-naphthoic acid	2480
<i>Hydroxyamines</i>	
<i>o</i> -Aminophenol	1670
<i>o</i> -Phenylenediamine	1820
3-Aminopropanol	1218



The butaneboronate and benzeneboronate derivatives were used to distinguish between the two isomeric hydroxy-salicylanilides, 3'-chloro-4'-(*p*-chlorophenoxy)-3-hydroxy-5-iodosalicylanilide and 3'-chloro-4'-(*p*-chlorophenoxy)-5-hydroxy-3-iodosalicylanilide by MS ²⁷¹. The two isomers formed different types of boronate derivatives which had clearly different mass spectra. The diol derivative had as its base peak the ion m/e ($272 + R_1$) which was absent in the mass spectra of the hydroxyamide derivative.

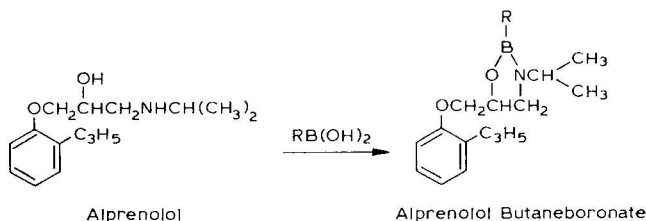


Salmefamol and labetalol (substituted propanolamines) form a bis(boronate) derivative when reacted with excess butaneboronic or benzeneboronic acid in anhydrous dimethylformamide for twenty minutes at room temperature⁵⁸. The boronate derivatives gave a good separation of the possible diastereoisomeric forms by GC but the quantitative results were found to be unreliable due to on-column decomposition.



Cyclic Boronate Derivative of Salmefamol

Alprenolol contains a propanolisopropylamine side chain which can be converted to its butaneboronate derivative by either heating at 80° for 2 h with excess butaneboronic acid²⁷² or by co-injection with a solution of butaneboronic acid in dimethylformamide^{193,273}. Butaneboronate formation used in conjunction with GC-EI-MS was useful for the identification of the drug and its metabolites in biological fluids, as several of the metabolites formed either retained the side chain composition intact or contained other bifunctional groups as a result of enzymatic modifications.

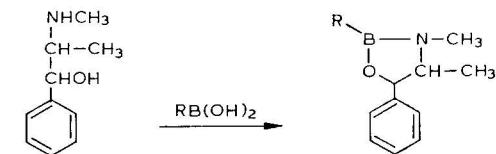


Alprenolol

Alprenolol Butaneboronate

Hydroxyamines and hydroxyacids which have been separated as their boronate derivatives by GC are summarized in Table 25.

10.2.7. Catecholamines. The catecholamines can be derivatized by a 1.5–2.0 mole excess of boronic acid in pyridine or dimethylformamide overnight at room temperature^{224,274}. Acetone is unsuitable as a solvent for the reaction due to the formation of secondary products, identified as oxazolidines in the case of secondary β -hydroxyamines. Yields were not always quantitative under these conditions²⁷⁴ and catecholamines with remaining unblocked polar groups (*e.g.*, synephrine, neosynephrine, octopamine etc.) had unsatisfactory chromatographic properties. However, attempted trimethylsilylation of unprotected phenol groups resulted in cleavage of the butaneboronate from the β -hydroxyamine function. The formation of boronate derivatives enabled a separation of the diastereoisomeric pair ephedrine and pseudo-



Ephedrine

Ephedrine Boronate Derivative

(61)

TABLE 25

BORONATE DERIVATIVES USED FOR THE GAS CHROMATOGRAPHY OF HYDROXY-AMINES AND HYDROXYACIDS

<i>Compound</i>	<i>Boronate derivative</i>	<i>Comments</i>	<i>Ref.</i>
Allothreonine methyl ester	BuB	I, EI-MS	211
Alprenolol (and metabolites)	BuB	GC, EI-MS	193
			272
			273
2-Amino-2-methylpropanol	BuB	I, EI-MS	211
<i>o</i> -Aminophenol	BuB	I, EI-MS	211
2-Aminopropanol	BuB	I, EI-MS	211
3-Aminopropanol	BuB	I, EI-MS	211
	BuB	I, EI-MS	224
Cannabidiolic acid	MeB, BuB, BenzB	I, EI-MS	270
Cannabinolic acid	MeB, BuB, BenzB	I, EI-MS	270
Ethanolamine	BuB	I, EI-MS	211
3-Hydroxybutyric acid	BuB	I, EI-MS	211
<i>cis</i> + <i>trans</i> -2-Hydroxycyclohexanecarboxylic acid	BuB	I, EI-MS	211
	BuB	I, EI-MS	224
2-Hydroxyisobutyric acid	BuB	I, EI-MS	211
4-Hydroxy-3-methoxymandelic acid	BuB	I, EI-MS	211
2-Hydroxymyristic acid	BuB	I, EI-MS	211
2-Hydroxy-1-naphthoic acid	BuB	I, EI-MS	211
2-Hydroxy-3-naphthoic acid	BuB	I, EI-MS	211
3-Hydroxypropionic acid	BuB	I, EI-MS	211
Labetalol	BuB, BenzB	GC	58
Lactic acid	BuB	I, EI-MS	224
	BuB	I, EI-MS	211
Mandelic acid	MeB, BuB, BenzB, CHB	I, EI-MS	211
	BuB	I, EI-MS	224
3-Methoxysalicylic acid	BuB	I, EI-MS	211
4-Methoxysalicylic acid	BuB	I, EI-MS	211
6-Methoxysalicylic acid	BuB	I, EI-MS	211
Phenylpropanolamine	MeB, BuB, BenzB, CHB	I, EI-MS	274
Phenylpyruvic acid	BuB	I, EI-MS	211
	BuB	I, EI-MS	224
Propylcannabinolic acid	MeB, BuB	I, EI-MS	270
Propyl- Δ^1 -tetrahydrocannabinolic acid	MeB, BuB	I, EI-MS	270
Salicylic acid	BuB	I, EI-MS	224
	MeB, BuB, BenzB, CHB	I, EI-MS	211
Salicylanilide	BuB, BenzB	GC	271
3'-Chloro-4'-(<i>p</i> -chlorophenoxy)-3-hydroxy-5-iodosalicylanilide	BuB, BenzB	EI-MS	271
3'-Chloro-4'-(<i>p</i> -chlorophenoxy)-3-hydroxy-5-iodosalicylanilide	BuB, BenzB	EI-MS	271
Salmefamol	BuB, BenzB	GC	58
Serine methyl ester	BuB	I, EI-MS	211
Tartaric acid	BuB	I, EI-MS	211
	BuB	I, EI-MS	224
Δ^1 -Tetrahydrocannabinolic acid	MeB, BuB, BenzB	I, EI-MS	270
<i>o</i> -Thymotic acid	BuB	I, EI-MS	224
	BuB	I, EI-MS	211

ephedrine to be achieved by GC^{224,274}. The retention index values for some butane-boronate catecholamine derivatives are summarized in Table 26. The mass spectra of the catecholamine boronate derivatives have very favorable properties, characterized by strong molecular ions and a few prominent fragment ions indicating the substituents attached to the oxazaboralidine ring^{224,274,275}.

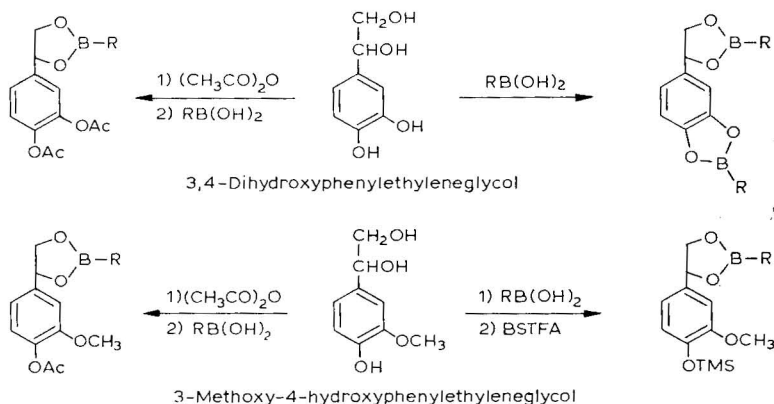
TABLE 26

RETENTION INDEX VALUES FOR SOME CATECHOLAMINE BUTANEBORONATE DERIVATIVES ON OV-17

6 ft. glass column of 1% OV-17 on Gas-Chrom Q (100–120 mesh).

<i>Catecholamine</i>	<i>Column temperature (°C)</i>	<i>Retention index</i>
β -Hydroxyphenethylamine	140	1799
Norpseudoephedrine	140	1774
Phenylpropanolamine	140	1776
Pseudoephedrine	140	1782
Ephedrine	140	1796
Octopamine	170	2218
4-Deoxynoradrenaline	170	2203
Synephrine	170	2185
Phenylephrine	170	2171
Normetanephrine	190	2315
Metanephrine	190	2270
Norepinephrine	190	2478
Epinephrine	190	2438
3,4-Dihydroxynorephedrine	190	2450
Isoprenaline	190	2512

The catecholamine metabolite 3,4-dihydroxyphenylethyleneglycol (DHPG) was converted to its bis(boronate) derivative in twenty minutes at room temperature with methaneboronic or butaneboronic acid in 2,2-dimethoxypropane^{276,277}. The bis(boronate) derivative was stable in solution for at least twenty-four hours at room temperature. The phenolic groups can also be acylated in aqueous solution and after extraction the ethyleneglycol function converted to its methaneboronate or butaneboronate derivative by co-injection of the acylated DHPG extract and boronic acid into the GC²⁷⁸. The reaction in solution or by co-injection proceeds to the same extent but a calibration curve covering the range 3–30 μ g was linear but did not pass through the origin. 3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG) can be converted to its methaneboronate or butaneboronate derivative by reaction in 2,2-dimethoxypropane as solvent for 15 min at room temperature. Attempted silylation of the phenolic group with BSTFA resulted in a slow cleavage of the boronate group beginning after about one hour. The TMS derivative was prepared quantitatively without cleavage of the boronate group by sandwich injection of the boronate derivative and BSTFA into the gas chromatograph²⁷⁸. The phenol group can also be acylated in aqueous solution and after extraction into an organic solvent the boronate derivative of the ethyleneglycol group formed by co-injection of the extract and boronic acid into the gas chromatograph²⁷⁹. Formation of the acyl derivative, extraction and on-column boronation is recommended when a simultaneous analysis of DHPG and MHPG is required²⁷⁶.



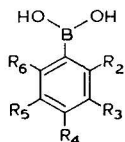
The catecholamines forming boronate derivatives suitable for their separation by GC are summarized in Table 27.

10.3. Boronic acids with electron-capturing properties

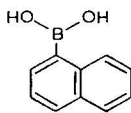
Boronic acids react specifically with bifunctional compounds to form derivatives stable to GC. The nature of the reaction provides some means of distinguishing this small group of compounds from the much larger pool of functionalized molecules in which they are found. The boronate reaction is specific as monofunctional compounds form derivatives which are not chromatographically stable. To take full advantage of this chemical specificity it is necessary to use a detector which is selective for the boronate derivatives and able to distinguish them from the general organic background. This combination of chemical specificity, chromatographic separating power and detector selectivity should provide a direct method of analyzing bifunctional compounds in complex mixtures (*e.g.*, physiological fluids) with the minimum of sample manipulation.

Hetero-element detectors have been developed for the selective detection of nitrogen, phosphorus and sulfur containing compounds. The scope of these detectors has been extended to embrace boron. The alkali flame ionization detector was adapted to the determination of sugar boronates²⁴⁹ and steroid boronates²⁵⁵ and the flame photometric detector to the determination of volatile boron hydrides²⁸⁰. Both detectors showed similar sensitivity with detection limits about 50-fold better than obtained with the universal flame ionization detector. This corresponds to a detection limit of about 10^{-8} g for the butaneboronate derivative of $2\beta,3\beta$ -dihydroxy- 5α -cholestane²⁵⁵. The selectivity of the detector response towards boron compared to potential interfering organic compounds was not determined. With the rubidium bead NPD under conditions optimized for boron, only a poor selective response for boron could be obtained. For the benzenboronate derivative of pinacol, the selective enhancement of the signal to boron over a hydrocarbon internal standard was about four-fold²⁸¹.

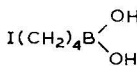
As an alternative approach to the specific detection of boron, boronic acids in which the organic substituent was modified to make it an electrophore have been prepared and used in conjunction with the selective and sensitive ECD²⁸¹⁻²⁸⁴. The structures and appropriate abbreviations for these boronic acids are given below. Since their synthesis in the laboratory, 2,4-dichlorobenzenboronic acid, 3,5-bis(trifluoro-



(I) → (VIII)



(IX)



(X)

(I) $R_2 = R_3 = R_4 = R_5 = R_6 = F$;	pentafluorobenzeneboronic acid ¹
(II) $R_3 = R_5 = R_6 = H, R_2 = R_4 = Cl$;	2,4-dichlorobenzeneboronic acid (2,4-DCBB)
(III) $R_3 = R_4 = R_5 = H, R_2 = R_6 = Cl$;	2,6-dichlorobenzeneboronic acid (2,6-DCBB)
(IV) $R_2 = R_4 = R_6 = H, R_3 = R_5 = Cl$;	3,5-dichlorobenzeneboronic acid (3,5-DCBB)
(V) $R_3 = R_5 = H, R_2 = R_4 = R_6 = Cl$;	2,4,6-trichlorobenzeneboronic acid (2,4,6-TCBB)
(VI) $R_2 = R_3 = R_5 = R_6 = H, R_4 = Br$;	4-bromobenzeneboronic acid (4-BrBB)
(VII) $R_2 = R_4 = R_5 = R_6 = H, R_3 = NO_2$;	3-nitrobenzeneboronic acid (3-NBB)
(VIII) $R_2 = R_4 = R_6 = H, R_3 = R_5 = CF_3$;	3,5-Bis-(trifluoromethyl)benzeneboronic acid (3,5-BTFMBB)
(IX) 1-Naphthaleneboronic acid	(NAPB)
(X) 4-Iodobutaneboronic acid	(4-IBuB)

methyl)benzeneboronic acid and 4-iodobutaneboronic acid have become available commercially from Lancaster Synthesis (St. Leonardgate, Lancaster, Great Britain) and in the U.S.A. through the Alfa Products Division (Ventron Corporation, Danvers, Mass., U.S.A.).

The selection of suitable electrophores for attachment to boron presents several problems of a chemical and chromatographic nature. The choice of substituents is limited in the main to organic groups containing halogen atoms, nitro groups or conjugated systems to provide the necessary high response to the ECD^{285,286}. As far as the halogens are concerned, the ECD has its highest response for iodine and declines through the series to fluorine, which is of course the reverse order of the volatility of their organic compounds on GC. To be of the widest possible use and particularly with high-molecular-weight compounds, it is of advantage if the boronic acid is capable of forming derivatives of high volatility. To provide good peak shape on GC and to reduce the possibility of column adsorption at low levels, the boronic acid should not contain polar groups in the organic substituent. Alkylboron compounds with halogen atoms on α , β or γ carbon atoms have poor hydrolytic and thermal stability. The thermally induced migration of halogens to boron with elimination of the organic group as an alkene takes place at temperatures likely to be employed for the separation of boronate derivatives by GC¹⁹⁸. The boron-acyl bond is moisture sensitive and chemically very reactive¹⁹⁶. The features discussed above influenced the choice of boronic acids (I) → (X) for evaluation as derivatizing reagents for GC with electron-capture detection.

All boronic acids except pentafluorobenzeneboronic acid can be used to derivatize a wide range of bifunctional compounds. Pentafluorobenzeneboronates were hydrolytically unstable and the presence of water (a product of the reaction to form the boronate derivative) resulted in elimination of pentafluorobenzene from the derivative²⁸³. Some bifunctional compounds did not produce derivatives with any boronic acid, these include bifunctional ketone compounds, thiol containing compounds, dicarboxylic acids and 1,3-substituted aromatic compounds. The boronate derivatives of aromatic carboxylic acids showed both solution and thermal degradation and are unsuitable for trace analysis. All derivatives were formed rapidly (within 15 min) at

TABLE 27

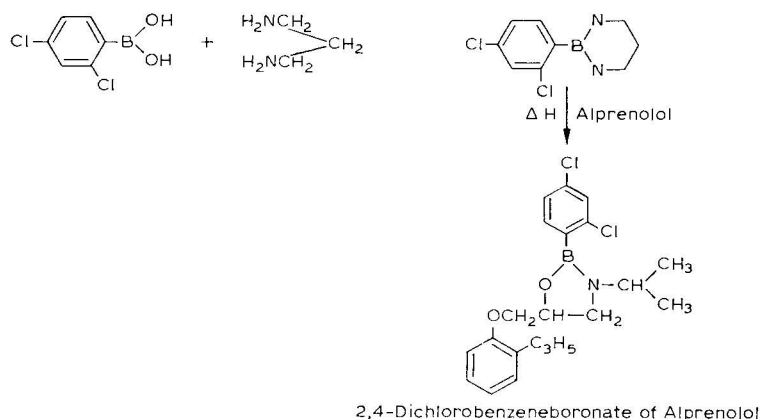
BORONATE DERIVATIVES USED IN THE GAS CHROMATOGRAPHY OF CATECHOLAMINES

<i>Catecholamine</i>	<i>Boronate derivative</i>	<i>Comments</i>	<i>Ref.</i>
4-Deoxynorepinephrine	BuB	I, EI-MS	274
3,4-Dihydroxynorephedrine	BuB	I, EI-MS	224
3,4-Dihydroxyphenylethyleneglycol	MeB, BuB	GC, EI-MS	276
	MeB, BuB	GC, EI-MS	277
Ephedrine	BuB, CHB, BenzB	GC, EI-MS	275
	BuB, CHB, BenzB	I, EI-MS	224
Epinephrine	BuB	I, EI-MS	274
	BuB	I, EI-MS	224
β -Hydroxy- β -phenylethylamine	BuB	I, EI-MS	224
	MeB, CHB, BenzB	I, EI-MS	274
	MeB, BuB, BenzB	GC, EI-MS	275
4-Hydroxyphenylethyleneglycol	MeB, BuB	GC, EI-MS	276
Isoprenaline	BuB	I, EI-MS	224
	BuB	I, EI-MS	274
Metanephrine	BuB	I, EI-MS	224
	BuB	I, EI-MS	274
3-Methoxy-4-hydroxyphenylethyleneglycol	MeB, BuB	GC, EI-MS	278
	MeB, BuB	GC, EI-MS	279
	MeB, BuB	GC, EI-MS	277
Methyl 2,3-dihydroxybenzoate	BuB	I, EI-MS	224
Methyl 3,4-dihydroxybenzoate	BuB	I, EI-MS	224
Neosynephrine	BuB	I, EI-MS	224
Norephedrine	BuB	I, EI-MS	224
	MeB, BuB, BenzB	GC, EI-MS	275
Norepinephrine	BuB	I, EI-MS	224
	BuB	I, EI-MS	274
Normetanephrine	BuB	I, EI-MS	224
			274
Norpseudoephedrine	BuB	I, EI-MS	274
	BuB	I, EI-MS	224
Octopamine	BuB	I, EI-MS	274
Phenylephrine	BuB	I, EI-MS	274
Pseudoephedrine	BuB	I, EI-MS	274
	BuB, CHB, BenzB	I, EI-MS	224
Synephrine	BuB	I, EI-MS	224
	BuB	I, EI-MS	274

room temperature in tetrahydrofuran as solvent by adding a molar quantity of the boronic acid to the bifunctional compound²⁸¹⁻²⁸⁴. Alternative methods of preparing boronate derivatives were investigated using 2,4-dichlorobenzeneboronic acid and 4-iodobutaneboronic acid²⁸⁷. These methods include on-column reaction by sandwich injection, extractive derivatization by using a solution of the boronic acid in an organic solvent to extract and derivatize bifunctional compounds in aqueous solution and a cartridge reactor consisting of a modified injection port liner packed with 2,4-dichlorobenzeneboronic acid coated onto Chromosorb W into which test solutions were injected in the usual way. The sandwich injection technique was evaluated for the organic acids which showed signs of solution degradation. Poor reproducibility was obtained with salicylic acid, anthranilic acid and mandelic acid as with the solu-

tion technique. Many of the boronate derivatives were hydrolytically unstable and this limited the quantitative aspects of the extractive derivatization technique to those bifunctional compounds containing sterically crowded functional groups. Catechol and pinacol could be extractively derivatized in this way but other compounds such as salicylic acid, 1,3-propanediamine, *o*-aminophenol and 3-amino-1-propanol either did not react at all or gave a very low yield of derivative. The cartridge reactor technique had the same range of application and limitations as the sandwich injection technique. Using the cartridge reactor a linear calibration curve over the range 0.1–3.0 μg was obtained for catechol with the FID.

A method has been developed for the determination of alprenolol by an on-column transboration reaction using the 2,4-dichlorobenzeneboronate derivative of 1,3-propanediamine as the transboration reagent²⁸⁸. Use of the transboration reagent had the advantage that excess reagent could be vented through the ECD without the large disruption in the baseline obtained by direct injection of 2,4-dichlorobenzeneboronic acid. Alprenolol extracted from plasma could be detected at the 0.1 ng level and a linear calibration graph was obtained over the range 0.5–70 ng.



The reaction was rapid and selective as no peaks were obtained for a plasma blank and could be used routinely to monitor this drug in plasma. Initial studies provided little evidence for reaction between alprenolol and 2,4-dichlorobenzeneboronic acid in solution and the balance of probability favors the observation that this reaction either with the boronic acid or by transboration occurs principally in the injection port of the gas chromatograph.

All boronate derivatives investigated had good peak shape on GC. The 3-nitrobenzeneboronate derivatives exhibited poor peak shape on OV-17 but could be chromatographed as symmetrical peaks on OV-225 (ref. 282). Several boronate derivatives were decomposed on stainless steel columns and the use of nickel or glass columns was recommended for their separation^{281–283}. Relative retention times for the chlorobenzeneboronate derivatives are summarized in Table 28, for the 3,5-bis(trifluoromethyl)benzeneboronate and benzeneboronate derivatives in Table 29 and for the 4-iodobutaneboronate and 4-chlorobutaneboronate derivatives in Table 30. A comparison of the relative volatility of the boronate derivatives, Table 31 illustrates some interesting features. The 3,5-bis(trifluoromethyl)benzeneboronates are re-

TABLE 28

RELATIVE VOLATILITY OF CHLOROBENZENE BORONATES

90 × 0.2 cm I.D. nickel column of 1% OV-17 on Gas-Chrom Q (100–120 mesh). Nitrogen flow-rate 60 ml min⁻¹. Compounds not forming boronate derivatives: 2,3-butanedione, 2,4-pentanedione, 2,5-hexanedione, 3-hydroxy-2-butanone, propane-1,3-dithiol; 2-anilinoethanol, 2-amino-2-hydroxy-methyl-1,3-propanediol, ethylenediamine, methylguanidine; isophthalic acid, *trans*-1,2-cyclobutanedicarboxylic acid, oxalic acid, 2,3-pyridinecarboxylic acid, pyruvic acid, gallic acid, isoleucine, succinic acid, citric acid, malonic acid; pentane-1,5-diol, sorbitol; *m*-phenylenediamine, resorcinol, 3-aminophenol. Compounds forming 4-iodobutaneboronates but not 2,4-DCBB: 3-hydroxypropionic acid, 1,2-; 5,6-dianhydrogalactitol. Compounds forming 2,4-DCBB but not 4-iodobutaneboronates: 1,3-propanediamine.

Compound	2,4-DCBB	3,5-DCBB	2,6-DCBB	2,4,6-TCBB	Column temperature (°C)
Ethyleneglycol	0.40	0.32	0.32	0.51	140*
Pinacol	0.47	0.40	0.56	0.96	140
1,3-Propanediol	0.75	0.71	0.55	0.95	140
1,4-Butanediol	1.26	1.15	0.93	1.58	140
1,3-Cyclopentanediol	1.59	1.45	1.55	2.35	140
<i>cis</i> -1,2-Cyclohexanediol	2.54	2.13	2.41	3.93	140
Lactic acid	0.71	0.54	0.47	—	140
1,3-Propanediamine	0.90	1.30	—	—	140
3-Amino-1-propanol	0.79	1.30	0.78	—	140
2-Amino-1-butanol	0.70	1.00	0.86	—	140
Catechol	2.36	1.89	1.87	3.02	140
Phenyl-1,2-ethanediol	0.41	0.35	0.38	0.54	210**
<i>o</i> -Phenylenediamine	0.71	0.85	0.58	0.90	210
<i>o</i> -Aminophenol	0.32	0.43	0.33	0.52	210
D,L-Mandelic acid	0.63	0.53	0.53	0.68	210
Salicylic acid	0.64	0.64	0.50	0.67	210
Anthranilic acid	1.61	—	1.93	2.95	210

* Internal standard C₂₀, retention time = 5.7 min.

** Internal standard C₂₈, retention time = 5.6 min.

markedly volatile having retention times significantly shorter than the benzeneboronates²⁸¹. The 4-iodobutaneboronates have retention times approximately 1.8 times those of the benzeneboronates²⁸⁴. The 3-nitrobenzeneboronates and naphthaleneboronates have inconveniently long retention times for general use in GC^{282,283}.

The magnitude of the ECD response is remarkably temperature dependent and this parameter should be optimized for the comparison of detector response for different derivatives of a compound²⁸⁹. The minimum detectable quantity of pinacol as its boronate derivatives at their optimum detector temperatures are compared in Table 32²⁸¹. With the exception of naphthaleneboronate all derivatives show a useful ECD response. The benzeneboronate derivative shows a moderate detector response which is enhanced by the introduction of halogen atoms. The position of the chlorine substituent effects the magnitude of the detector response and the 2,4-dichlorobenzeneboronate derivative was the most sensitive of the chlorobenzeneboronic acids evaluated. The 3,5-bis(trifluoromethyl)benzeneboronate derivative gave its maximum response at a low detector temperature and the detection limit was raised by a factor of 17 when the detector temperature was increased by 170°. High detector temperatures

TABLE 29

RETENTION TIME DATA FOR THE 3,5-BTFMB AND BenzB DERIVATIVES OF SOME REPRESENTATIVE BIFUNCTIONAL COMPOUNDS

Compound	Derivative		Column temperature (°C)
	BB	3,5-BTFMBB	
Ethylene glycol	1.00	0.33	80*
Pinacol	1.73	0.41	80
1,3-Propanediol	2.60	0.97	80
Lactic acid	2.39	—	80
1,4-Butanediol	1.25	0.47	100**
cis-1,2-Cyclohexanediol	2.84	0.79	100
1,3-Propanediamine	1.21	0.52	100
3-Amino-1-propanol	1.16	0.47	100
2-Amino-1-butanol	1.08	0.42	100
Catechol	2.45	0.74	100
Phenyl-1,2-ethanediol	0.67	0.19	160***
o-Phenylenediamine	1.43	0.57	160
o-Aminophenol	0.64	0.25	160

* Internal standard C₁₄, retention time = 3.0 min.** Internal standard C₁₆, retention time = 3.8 min.*** Internal standard C₂₂, retention time = 4.2 min.

are often preferred for the analysis of biological samples as this reduces detector contamination to the minimum.

The pinacol boronate derivatives are compared in terms of hydrolytic stability in Table 33. These data were gathered by partitioning an organic solution of the pinacol boronate against an aqueous sodium hydroxide solution for a fixed time interval

TABLE 30

RETENTION TIME DATA FOR 4-IBuB AND 4-CIBuB DERIVATIVES OF SOME REPRESENTATIVE BIFUNCTIONAL COMPOUNDS

Nickel column 90 × 0.2 cm I.D. packed with 1% OV-17 on Gas-Chrom Q (100–120 mesh). Nitrogen flow-rate = 60 ml min⁻¹.

Compound	Derivative		Column temperature (°C)
	4-Chlorobutane-boronate	4-Iodobutane-boronate	
1,2-Ethanediol	0.05	0.17	120*
Pinacol	0.09	0.26	120
1,3-Propanediol	0.10	0.31	120
1,3-Butanediol	0.21	0.60	120
Lactic acid	0.13	0.45	120
3-Aminopropan-1-ol	0.13	0.48	120
2-Aminobutan-1-ol	0.14	0.45	120
Catechol	0.36	1.00	150**
cis-Cyclohexane-1,2-diol	0.29	0.87	150
o-Phenylenediamine	0.59	1.47	200***
Mandelic acid	0.52	1.28	200
Salicylic acid	0.52	1.30	200

* Internal standard C₁₉, retention time = 9.6 min.** Internal standard C₂₀, retention time = 3.7 min.*** Internal standard C₂₄, retention time = 1.9 min.

TABLE 31

RELATIVE VOLATILITY OF THE BORONATE DERIVATIVES

<i>Boronic Ester</i>	<i>Relative volatility</i>
3,5-Bis(trifluoromethyl)benzeneboronates	0.3 ± 0.05
Benzeneboronates	1.0
4-Iodobutaneboronates	1.8 ± 0.5
4-Bromobenzeneboronates	3.9 ± 0.8
2,6-Dichlorobenzeneboronates	4.3 ± 2.0
2,4-Dichlorobenzeneboronates	4.7 ± 1.7
3,5-Dichlorobenzeneboronates	5.0 ± 1.1
2,4,6-Trichlorobenzeneboronates	6.9 ± 1.8
3-Nitrobenzeneboronates	11.7 ± 3.4
Naphthaleneboronates	18.5 ± 4.6

TABLE 32

RELATIVE ELECTRON-CAPTURE DETECTOR SENSITIVITY OF THE PINACOL BORONATES²⁸¹

Detector A: Coaxial displaced cylinder type with a ^{63}Ni (8 mCi) source, operated in the pulse-modulated constant-current mode. Detector B: Coaxial cylinder type with a ^{63}Ni (30 mCi) source operated in the pulse mode with a pulse width of 4 μsec and a pulse period of 200 μsec .

<i>Pinacol boronate</i>	<i>Optimum detector temperature ($^{\circ}\text{C}$)</i>	<i>Detector type</i>	<i>MDQ</i> ($\times 10^{-12}$ g)
2,4-Dichlorobenzeneboronate	325	B	2.0
	380	A	4.0
4-Bromobenzeneboronate	350	B	3.0
3,5-Bis(trifluoromethyl)benzeneboronate	180	A	3.0
2,4,6-Trichlorobenzeneboronate	380	A	4.0
3-Nitrobenzeneboronate	300	B	4.0
3,5-Dichlorobenzeneboronate	380	A	11.0
	325	B	9.0
4-Iodobutaneboronate	325	B	16.0
2,6-Dichlorobenzeneboronate	380	A	18.0
Benzeneboronate	200	B*	150.0
Naphthaleneboronate	350	B	2550.0

* This detector temperature is not optimum.

TABLE 33

RELATIVE HYDROLYTIC STABILITY OF PINACOL BORONATES

Contact time for hydrolysis experiment = 8.0 min; see ref. 281 for experimental details.

<i>Pinacol boronate</i>	<i>Percent hydrolysis</i>	
	<i>1 N NaOH</i>	<i>3 N NaOH</i>
Benzeneboronate	5	30
4-Iodobutaneboronate	5	22
4-Bromobenzeneboronate	15	31
2,6-Dichlorobenzeneboronate	24	—
3,5-Bis(trifluoromethyl)benzeneboronate	25	35
3,5-Dichlorobenzeneboronate	26	65
2,4,6-Trichlorobenzeneboronate	72	92
2,4-Dichlorobenzeneboronate	76	94

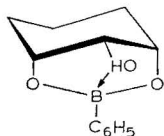
and then determining the percent of derivative which had been hydrolyzed. As can be seen the boronic acids show a wide range of hydrolytic stability varying from relatively stable to easily hydrolyzable.

An overview of the properties of the electron-capturing boronic acids lead to the recommendation that the four boronic acids, 2,4-dichlorobenzeneboronic acid, 4-bromobenzeneboronic acid, 4-iodobutaneboronic acid and 3,5-bis(trifluoromethyl)-benzeneboronic acid embodied a diverse range of advantages and some disadvantages which makes them the reagents of choice from the boronic acids tested for initial experimentation²⁸¹.

10.4 Miscellaneous chromatographic applications of boronic acids

Carbohydrates and acetylated carbohydrate derivatives were separated by paper electrophoresis in sulfonated benzeneboronic acid buffers at neutral pH²⁹⁰. The acetylated carbohydrates could not be separated in conventional buffer systems due to deacetylation or acyl migration occurring in alkali buffers and in acid buffers the derivatives had too low a mobility for adequate separation. Sugars with pyranosidic *cis*-1,2-diols and *cis*-1,3-diols showed little increase in mobilities compared to the furanosidic *cis*-1,2-diols which showed high mobilities. The spread of mobilities for the monosaccharides in the sulfonated benzeneboronic acid buffer enabled good separations to be obtained. Increased mobilities for some monosaccharides were also found for *ortho*- and *meta*-nitrobenzeneboronic acid complexes but the poor water solubility of these reagents rendered them unsuitable.

The addition of benzeneboronic acid to paper chromatographic solvents specifically enhances the mobilities of compounds possessing diol systems which form stable benzeneboronate derivatives²⁹¹⁻²⁹⁴. With monosaccharides the most stable derivatives (*i.e.* those with the highest mobility) were formed between benzeneboronic acid and monosaccharides with *cis*-1,3 axial hydroxyl groups stabilized towards hydrolysis by the intervening equatorial hydroxyl group. The alditols (except glycerol) all form



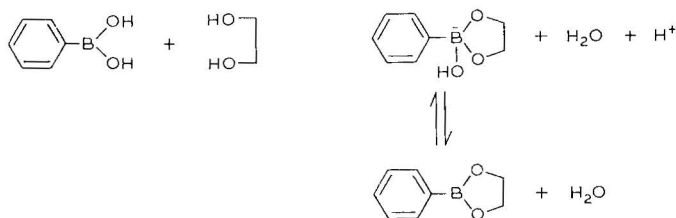
strong boronate complexes which enables the reduced form of the sugar to be easily separated from its parent. The configuration of 2-C-methyl-L-arabinose and L-ribose was assigned in part based on their ability to form complexes of enhanced mobility with benzeneboronic acid²⁹⁴. The paper chromatographic mobilities of cardenolides and bufadienolides with *cis*-1,3- and *cis*-1,2-diols were increased in the presence of benzeneboronic acid and at higher concentration by dibenzeneboronic acid as well. Diols containing a tertiary hydroxyl group formed particularly stable complexes²⁹³.

Phenanthreneboronic acid has been evaluated as a selective fluorescent reagent for the analysis of bifunctional compounds by HPLC and HPTLC²⁹⁵. Only compounds with sterically hindered hydroxyl groups yielded derivatives that were stable to reversed-phase HPLC or silica gel HPTLC. Hydrolysis of the derivatives occurred in most of the examples studied (*e.g.*, 1,2-ethanediol, 3-amino-1-propanol, *o*-phenylenediamine, catechol, salicylic acid etc.). The phenanthreneboronate derivative of pinacol was stable to HPTLC and could be detected by its fluorescence at 385 nm

when excited at 313 nm down to the 0.6-ng level. The fluorescence intensity decreased with time in the form of a shallow curve possibly due to either a slow chemical reaction on the plate or due to oxygen quenching. The insect moulting hormones, ecdysone and ecdysterone, were separated as their phenanthreneboronate derivatives by HPTLC and qualitatively identified by fluorescence measurement. The method was sufficiently selective for the detection of ecdysones in crude insect samples.

10.5. Immobilized boronic acid phases for liquid column chromatography

Ion-exchange chromatography of sugars in the form of their anionic boronate complexes is a well established technique for their separation and analysis. The aromatic boronic acids are also capable of complexing with sugars in a manner similar to boric acid^{296,297}. The presence of the aromatic group in the boronic acid makes for a simplification in the complexation reaction as only 1:1 complexes can be formed.

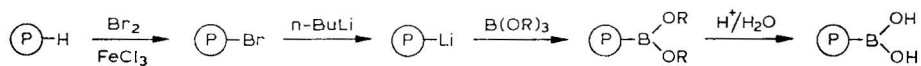


Experimental evidence for complex formation is derived from the increase in acidity observed when a solution of benzeneboronic acid is added to certain diols with the correct spatial arrangement for complex formation²⁹⁸. The formation of the anionic complex is favored in alkaline solution and the pH dependence of the equilibria between complex formation and dissociation enables a chromatographic separation to be devised. As benzeneboronic acids are Lewis acids, their acidity is affected by the electron density on the boron atom and the anionic boronate complex should be stabilized by electron-withdrawing groups in the aromatic ring. For example, the pH value for 100% formation of the benzeneboronate complex of D-glucose and D-fructose is close to pH 9.0 whereas for the corresponding 3-nitrobenzeneboronate complex it is close to pH 7.9 (ref. 299).

Immobilization of the boronic acid group by incorporation or attachment to a polymer network provides chromatographic materials which were used to isolate or separate bifunctional compounds. The sample is passed through the column material at a controlled pH favoring formation of the anionic complex which then remains attached to the polymer while other non-complexed compounds are washed away (some non-specific adsorption of compounds to the polymer network may also occur). The complexation reaction is reversible by adjustment of the pH in a stepwise fashion or with a continuous pH gradient for separation based on the stability of the anionic complex or by elution at acid pH, at which all anionic complexes are dissociated for isolation purposes. In practice, the binding capacity of the boronic acid resin or gel is highly influenced by the ionic strength of the solution and also by the amount of interfering non-bifunctional compounds. To date, most work has concerned the use of boronic acid resins or gels for the separation of sugars and nucleosides (via the ribose substituent) or catecholamines in aqueous media including biological fluids such as serum, amniotic fluid and urine.

Ion-exchange resins with boronic acid groups were first prepared by Solms and Deuel³⁰⁰. Acid-catalyzed condensation polymerization of *m*-aminobenzeneboronic acid, *m*-diaminobenzene and formaldehyde was used. The resulting material was treated with formic acid-hydrochloric acid or with pyridine and acetic anhydride to cap the free amine groups. An alternative resin was prepared by condensation of formaldehyde and *m*-aminobenzeneboronic acid with the weakly basic anion-exchange resin Duolite A114. Resins of the above type were generally chromatographically inefficient and contained residual ion-exchange sites but could be used to separate simple sugar mixtures.

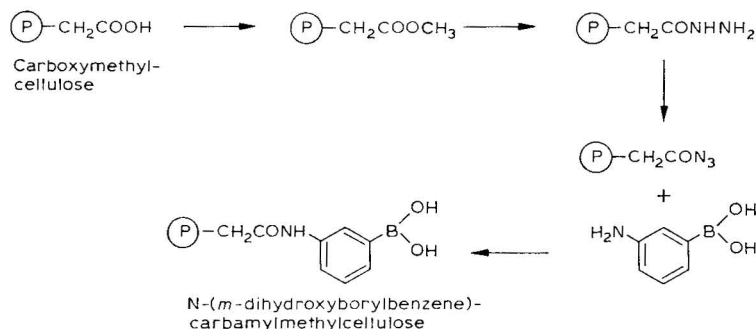
The successful synthesis of *p*-vinylbenzeneboronic acid led to the production of polymeric materials either by catalyzed self condensation or by co-polymerization with styrene or diallylmalate³⁰¹⁻³⁰⁴. These materials were obtained in the form of air stable powders or "popcorn" polymers but were not tested specifically as chromatographic materials. It was noted however that the polymeric boronic acid materials would bind 2-aminoethanol and *o*-diaminobenzene³⁰². The iminodiethyl derivative of 4-vinylbenzeneboronic acid was polymerized with a mixture of divinylbenzene, ethylvinylbenzene and azobisisobutyronitrile (free radical catalyst) to form a lightly cross-linked gel which swelled in alkaline aqueous solution and thus allowed monosaccharides to enter the gel matrix³⁰⁵. The boronic acid gel was successfully used to separate simple mixtures of monosaccharides and the effect of pH and temperature on retention factors was determined. The use of the polymer to improve the yield of D-fructose from D-glucose (the former resulting from the action of alkali on the latter) was also demonstrated using a closed system in which the product from a conversion reactor was passed continuously through the boronic acid column to selectively remove fructose from the reaction mixture and the eluent enriched in glucose fed back to the reactor. 4-Vinylbenzeneboronic acid has also been polymerized³⁰⁶ by (a) copolymerization with styrene; (b) copolymerization with N,N'-methylenebis(acrylamide); (c) copolymerization with acrylamide and N,N'-methylenebis(acrylamide) and (d) interstitial homopolymerization on porous polystyrene beads (Chromosorb 102). The boronic acid polymers were evaluated chromatographically for the separation of L-DOPA from L-tyrosine. Polymer (d) was found to be superior in performance to the other three polymers (a) → (c). At pH 8.0 the breakthrough capacity for a 21.0 × 1.0 cm column containing 5.36 g of polymer (d) was 120 ml (130 μmole g⁻¹) for L-DOPA compared to about 10 ml of solution for L-tyrosine. Elution of complexed L-DOPA and column regeneration was achieved by using 0.1 M acetic acid as the eluent. An investigation of a wide range of related hydroxyacids and phenols indicated that the column selectively complexed only *o*-dihydroxyaromatic compounds and that the binding of these compounds to the boronic acid resin was strongly pH dependent. Macroreticular porous polystyrene-divinylbenzene resins (e.g., Amberlite XE-305) can be functionalized to contain the boronic acid group by the route shown below³⁰⁷.



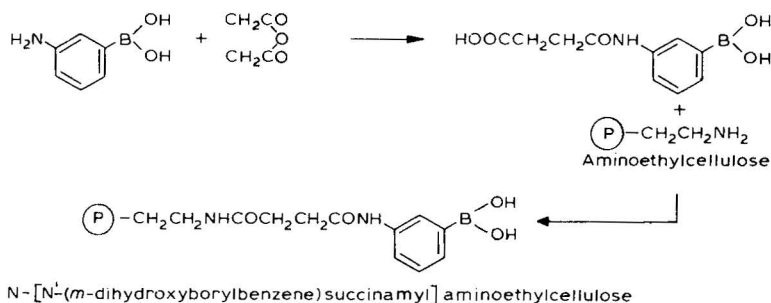
(P) = Polymer matrix

Polymers containing 1–2 mmole g^{-1} of boronic acid groups were prepared in this way and used in the solid phase synthesis of glycosides. The glycoside was coupled to the polymer in dry pyridine by azeotropic removal of water and was removed at the end of the synthesis by suspension in acetone–water (4:1). A similar polymer was used to separate *cis*-1,2-cyclohexanediol from its *trans* isomer by batchwise extraction. The *cis* isomer reacted selectively with the boronic acid resin and was then separated from the *trans* isomer which remained in solution by filtration³⁰⁸.

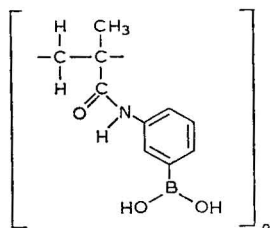
For the study of large biological molecules, the use of resins or gels with pores presenting restricted access to the boronic acid group could be a problem. For the analysis of polynucleotides, Weith *et al.*³⁰⁹ have described the synthesis of boronic acid groups attached to cellulose powder. In their synthesis, carboxymethylcellulose was converted to the azide form and coupled with *m*-aminobenzeneboronic acid to form *N*-(*m*-dihydroxyborylbenzene)carbamylmethylcellulose containing about 0.2 mmole $-\text{B}(\text{OH})_2 \text{ g}^{-1}$ of dry cellulose.



The material obtained in this way still contained a relatively high concentration of carboxylic acid groups (approximately 33% of the carboxymethyl groups were converted to the boronic acid) which was undesirable for some applications. In an alternative synthesis, *m*-aminobenzeneboronic acid was converted into *N*-(dihydroxyborylbenzene)succinamic acid and then condensed with aminoethylcellulose in the presence of *N*-cyclohexyl-*N'*- β -(4-methylmorpholinium)ethylcarbodiimide *p*-toluenesulfonate as the activating agent. The *N*-[*N'*-(*m*-dihydroxyborylbenzene)succinamyl] aminoethylcellulose material contained approximately 0.6 mmole of $-\text{B}(\text{OH})_2 \text{ g}^{-1}$ of cellulose corresponding to substitution of 60% of the amino groups present in the starting material³¹⁰. At neutral pH the remaining amine groups are not protonated and

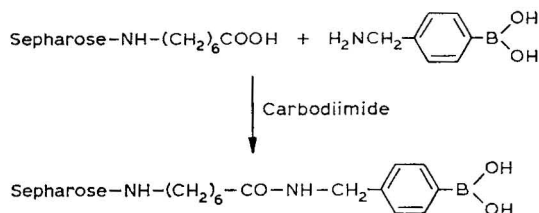


do not interfere in the formation of the boronate complexes. Columns of the above type when used to separate nucleosides showed a strong dependence of the elution order on the presence of a *cis*-glycol system in the sugar moiety, the pH of the eluting solvent, the ionic strength of the eluting solvent and the nature of the nucleoside base. Sugars which are bound most strongly are those with the highest proportion of *cis*-glycol groups especially if the glycol exist in a coplanar conformation. The retention of polynucleotides was found to be dependent on similar factors³⁰⁹⁻³¹¹.

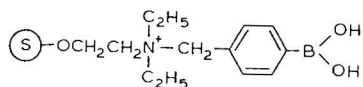


Schott has described the synthesis of a dihydroxyborylsubstituted methacrylic acid polymer with the general structure shown below³¹². Depending on the reaction conditions, the boron content of the polymers varied from 0.11 to 0.58 %. These polymers have been shown to be suitable for the separation of ribonucleosides from deoxyribonucleosides (not retained), ribonucleotides carrying 2'- or 3'-*cis*-diol groups from the corresponding deoxynucleoside 5'-phosphate and ribonucleoside 2'- or 3'-monophosphates, the separation of oligonucleotide mixtures and the separation of aminoacylated tRNA from unchanged tRNA³¹³. The capacity of the boronate gels seems to depend on the nature and particularly the chain length of the oligonucleotides. This difference probably arises from the reduced accessibility of the boronic acid groups in the interior parts of the gel matrix to molecules of large diameter. An improved method for the synthesis of polyacrylamide-boronate gel, based on the approach described by Weith *et al.*³⁰⁹, has been proposed by Hagen and Kuehn³¹⁴. Aminoethyl Bio-Gel P-150 was converted to the N-succinylaminoethyl derivative and coupled to *m*-aminobenzenboronic acid at pH 4.7 in the presence of L-ethyl-3(3-dimethylaminopropyl)carbodiimide. Under these conditions coupling was almost complete and gels containing between 0.9–1.1 mmole of $-B(OH)_2$ g⁻¹ were obtained. This boronic acid gel was used to separate ATP (retained through the *cis*-diol moiety on the ribose ring) from cyclic AMP which does not bind to the boronate gel.

p-Methylaminebenzenboronic acid has been attached to CH-Sepharose (a Sepharose derivative containing ϵ -aminocaproic acid residues with free carboxyl groups) activated by N-cyclohexyl-N'-[2-(4-morpholinyl)ethyl]carbodiimide *p*-toluenesulfonate³¹⁵. Gels prepared in this way contained 5–10 μ mole $-B(OH)_2$ ml⁻¹ of swollen Sepharose and were used to purify Subtilisin BPN', an extracellular serine

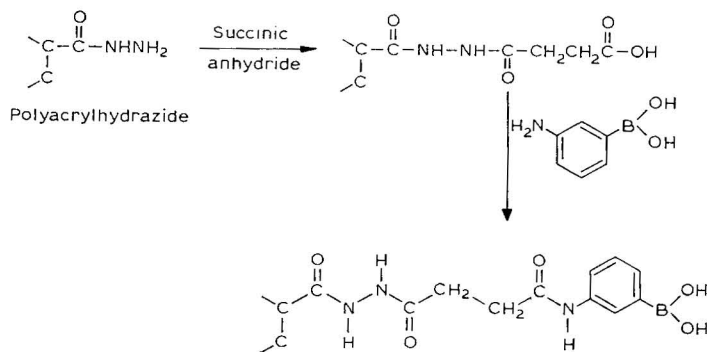


proteinase of "*Bacillus subtilis*". Binding was assumed to be through the functional groups of the amino acid constituents of the enzyme. A column of the boronate gel was used to provide a 42-fold purification of a culture medium extract. A 1.9-fold purification of a commercial source of the enzyme was obtained on the same column. Sephadex A-25 was converted to its N,N-diethyl-N-(*p*-methylbenzeneboryl)aminoethyl derivative and was evaluated for complexing with *cis*-diol compounds^{316,317}. The polymer was highly specific for the binding of polyols, carbohydrates, nucleosides and nucleotides over a wide pH range. The chromatographic behavior of carbohydrates was controlled by their structure and conformation which was also responsible for the different stabilities of the boronate anionic complexes generated^{317,318}.



The monosaccharides D-ribose, D-mannose, D-arabinose were separated by a 0.01 *M* [(C₂H₅)₃NH]HCO₃ buffer at pH 8.4, lactose, D-glucose and D-ribose with a 0.01 *M* sodium acetate buffer pH 6.5 and D-glucitol and D-fructose with the previous buffer at pH 5.0 (ref. 318). The same boronic acid gel was also used for the separation of nucleosides and mononucleotides³¹⁷, oligonucleotides³¹⁹ and in the isolation of pure aminoacylated nucleotides³¹⁹.

Polyacrylylhydrazide can be succinylated and then condensed with *m*-aminobenzeneboronic acid in the presence of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide to give a polyacrylamide boronic acid gel which has been extensively evaluated for the analysis of nucleosides³²⁰. The synthesis of the gel has been optimized by subsequent workers and described in detail³²¹. Compared to the synthesis by Uziel *et al.*³²⁰ a finer mesh polyacrylylhydrazide with a lower concentration of hydrazide groups (1.2 mmole g⁻¹ dry weight) was used as starting material to give a polymer gel with decreased



shrinkage when either the pH or the ionic strength of the eluent was changed. A synthetic mixture of nine nucleosides at the 10 nmole level each, had recoveries of 88–101 % when put on a polyacrylamide boronic acid gel column in an ammonium acetate buffer of pH 8.8 and eluted with 0.1 *M* formic acid. The columns could be regenerated and used many times by washing with formic acid and re-equilibrated before use with ammonium acetate buffer pH 8.8. The capacity limit of 0.8 ml of polyacrylamide boronic acid gel was established as 40–50 μ moles of nucleosides at

pH 8.5 to 9.4 (ref. 322). It seems likely that complex formation between the diol of the nucleosides and the boronic acid group is not the only process controlling the binding of the nucleosides. The order of elution from the column places nucleosides with an intrinsic negative charge (exclusive of boronic acid–diol complexation) at the front of the elution pattern and those nucleosides that are cations or potential cations at the rear³²⁰. For the common nucleosides found in biological fluids, pseudouridine is the least tightly bound and its retention is strongly influenced by the pH of the solution used to load the sample on the column and by the concentration of other substances present in urine besides the ribonucleosides^{320,322,323}. A complete analytical scheme comprising preliminary isolation of the nucleosides from biological fluids using a polyacrylamide boronic acid gel column with elution and collection of the nucleoside fraction and subsequent separation and analysis by HPLC has been described and used for the analysis of nucleosides in urine, blood, amniotic fluid and tRNA hydrolysates^{321,322,324}. A similar boronic acid gel was used as a preliminary isolation procedure for 5-fluorouridine in urine³²⁵ and 5-fluoro-2'-deoxyuridine in plasma³²⁶ which were determined by capillary column GC after derivatization. The polyacrylamide boronic acid gel has also been used to analyze cysteinyl dopas, dopamine and catecholamines which were separated into three separate fractions using a stepwise pH gradient³²⁷. Adsorption of all catecholic compounds on the immobilized benzeneboronate gel takes place at neutral or alkali pH (*i.e.* 8.0 or higher) and at slightly lower pH the extent of adsorption becomes dependent on the compound concerned. The gel was specific for the catecholamine structure as neither tyrosine nor 3-methoxytyramine were adsorbed even at high pH.

Polymers bearing structural analogies to biological receptors were prepared containing a boronic acid group locked into a fixed position in a polymer cavity³²⁸. Vinylboronic acid was condensed with a template molecule, *p*-nitrobenzene- α -D-mannopyranoside and then polymerized with a co-monomer (also a cross-linking agent) in the presence of an inert solvent to obtain a macroporous polymer³²⁹. High cross-linking ensured a rigid polymer with fixed cavities that contain the boronic acid group (after selective removal of the template) locked in a fixed stereochemical arrangement. The polymer was used in HPLC to analyze the optical isomers of the template with very high specificity (separation factors $\alpha = 1.05$ –2.32). Unfortunately, the columns themselves were inefficient as measured by their plate height and also showed excessive tailing which tended to offset the advantages gained by the high separation factors. The high specificity of the polymers was demonstrated by the lack of resolution of racemates other than the template.

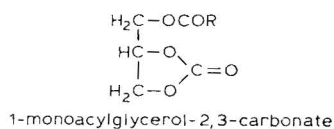
11. MISCELLANEOUS REAGENTS FOR THE DERIVATIZATION OF BIFUNCTIONAL COMPOUNDS

Melatonin reacts with pentafluoropropionic anhydride under mild conditions to form a 3,3-spirocyclic indole derivative^{330,331}. The ethyl and propyl amide analogues



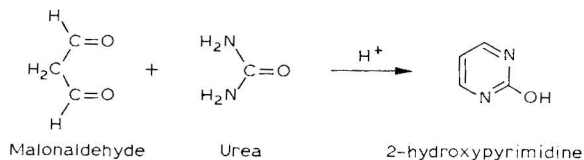
of melatonin were also cyclized but the isobutyryl amide gave a low yield of the cyclic derivative and the pivaloyl amide underwent displacement of the pivaloyl group with replacement by a pentafluoropropionyl group. The structures of all derivatives were confirmed by physical means³³⁰. The melatonin derivative could be detected in plasma at the 1 pg ml^{-1} level by negative chemical-ionization MS^{331,332}.

The mass spectral properties of a miscellaneous series of cyclic sulfite and carbonate derivatives have been described³³³. The cyclic carbonate derivatives were formed by reaction with phosgene in yields of 50–70%, some of which were purified by GC. Cyclic carbonate derivatives have been used for the separation by GC and identification by MS of the 1-monoacyl-glycerol-2,3-carbonates^{334,335}. The derivatives were prepared by the cyclization of the glycerol 1- β,β,β -trichloroethylcarbonate in pyridine at 80° (ref. 334). In an alternative synthesis, 1-O-benzylglycerol was converted to its 2,3-carbonate derivative by treatment with potassium hydrogen carbonate and diethylcarbonate.

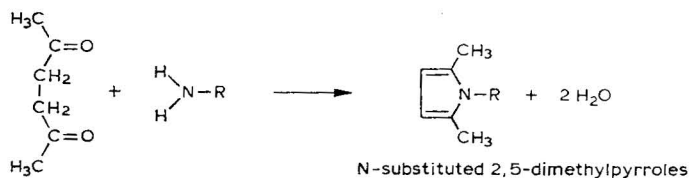


The mass spectra of the 1-monoacylglycerol-2,3-carbonates were characterized by the presence of a molecular ion and daughter fragment ions of high abundance identifying the acyl group and the presence of the cyclic carbonate group³³⁵.

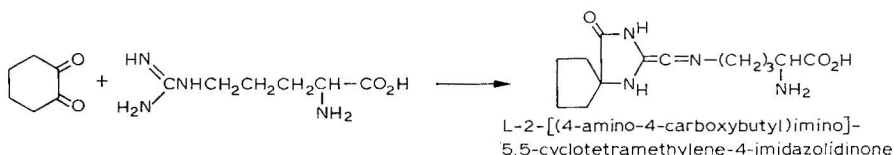
Malonaldehyde was converted to 2-hydroxypyrimidine in approximately 90% yield by reaction with excess urea in acid solution³³⁶. The hydroxypyrimidine was isolated by ion-exchange chromatography and converted to its TMS ether derivative for GC with identification by MS.



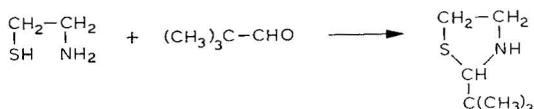
Primary aliphatic and aromatic amines react to form the N-substituted 2,5-dimethylpyrrole derivatives quantitatively with 2,5-hexanedione by heating the amine with a five-fold excess of 2,5-hexanedione in ether at 60° for 30 min in a vessel protected from light with nitrogen bubbling through the solution³³⁷. Aromatic amines react much slower than aliphatic ones and sterically hindered amines like 2,6-dimethylaniline require heating for 2 h for complete reaction and *tert*-butylamine does not react quantitatively under any conditions. Diamines such as *p*-phenylenediamine are



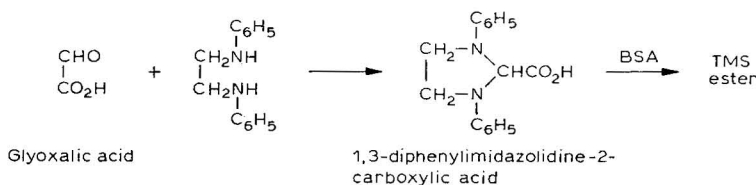
rapidly converted to the monoamine derivative and only slowly to the di derivative. The presence of water in the reaction mixture lowers the yield of derivatives. Under the influence of light, oxygen or acid the reaction mixture deteriorates producing discoloration and a low yield of derivative. Arginine reacts with 1,2-cyclohexanedione in aqueous base to form a cyclic imidazolidinone derivative which was used for the detection of arginine residues in peptide digests by paper chromatography and electrophoresis³³⁸. A similar derivative was formed with benzil.



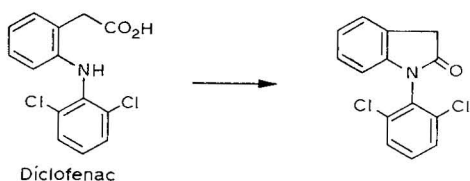
Aminothiols react rapidly at neutral pH and at room temperature with pivalaldehyde to form cyclic thiozolidine derivatives which are stable to GC³³⁹. The reaction was complete within 10 min when a small amount of basic ion exchanger in the bicarbonate form was added as a catalyst. Thiolamino acids and disulfides extracted from biological fluids could be separated by GC after methylation and condensation with pivalaldehyde.



Glyoxalic acid was converted to an imidazolidine-2-carboxylic acid derivative by treatment with N,N'-diphenylenediamine in pyridine at room temperature for 2 h which could be gas chromatographed as its TMS ester³⁴⁰.

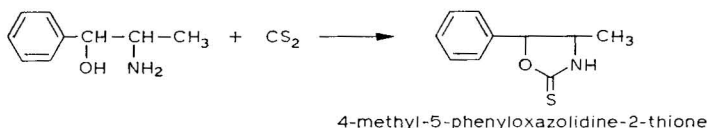


Diclofenac was converted to its indole derivative by heating at 70° for 75 min in a 0.5% (v/v) solution of sulfuric acid in 2,2,2-trifluoroethanol³⁴¹. The derivative was stable to GC and could be determined with an ECD at the nanogram level.



Phenylpropanolamine reacts with carbon disulfide at room temperature in a non-quantitative reaction to form 4-methyl-5-phenyloxazolidine-2-thione³⁴². The

derivative could be gas chromatographed with or without formation of its N-TMS derivative.



12. ABBREVIATIONS

AFID	Alkali flame ionization detector
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BenzB	Benzeneboronate
BrBB	Bromobenzeneboronate
BSA	N,O-Bis(trimethylsilyl)acetamide
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
BTFMBB	Bis(trifluoromethyl)benzeneboronate
BuB	Butaneboronate
t-BuB	<i>tert.</i> -Butaneboronate
CHB	Cyclohexaneboronate
CI	Chemical ionization
ClBuB	Chlorobutaneboronate
CMDMCS	Chloromethyldimethylchlorosilane
CMTMDS	1,3-Bis(chloromethyl)-1,1,3,3-tetramethyldisilazane
DCBB	Dichlorobenzeneboronate
DHPG	3,4-Dihydroxyphenylethyleneglycol
DMCS	Dimethyldichlorosilane
DMDAS	Dimethyldiacetoxysilane
DMMCS	Dimethylmethoxychlorosilane
DMSO	Dimethyl sulfoxide
DOPA	Dihydroxyphenylalanine
ECD	Electron-capture detector
EI	Electron impact
EPTD	Ethylphosphonothioic dichloride
FID	Flame ionization detector
FPD	Flame photometric detector
FR	Flow-rate
GC	Gas chromatography
HFAA	Hexafluoroacetylacetone
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
HMDS	Hexamethyldisilazane
<i>I</i>	Retention index according to Kováts
IBuB	Iodobutaneboronate
IR	Infrared spectroscopy
IS	Internal standard
MDQ	Minimum detectable quantity

MeB	Methaneboronate
MHPG	3-Methoxy-4-hydroxyphenylethyleneglycol
MS	Mass spectrometry
MTH	Methylthiohydantoin derivatives
MU	Methylene unit value
NAPB	1-Naphthaleneboronate
NBB	Nitrobenzeneboronate
NMR	Nuclear magnetic resonance spectrometry
NPD	Rubidium bead nitrogen-phosphorus detector
OR	Optical rotation
PAPTH	<i>p</i> -Phenylazophenylthiohydantoin derivatives
PFPTH	Pentafluorophenylthiohydantoin derivatives
PTH	Phenylthiohydantoin derivatives
tRNA	Transfer ribonucleic acid
TLC	Thin-layer chromatography
TMCS	Trimethylchlorosilane
TMDS	1,1,3,3-Tetramethyldisilazane
TMS	Trimethylsilyl ether/ester
UV	Ultraviolet spectroscopy

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14. SUMMARY

Only a few reagents capable of forming cyclic derivatives with bifunctional compounds have been described in the chromatographic literature. Some reagents are selective for a particular compound, whereas others such as the boronic acids are more generally applicable to a wide range of bifunctional compounds. All reagents provide a high degree of selectivity through the chemical discriminatory power of the reaction employed and some reagents have been developed which have high detector discriminatory power as well. These are of particular interest for the analysis of a few components (bifunctional compounds) in a complex matrix without the need for a tedious amount of sample clean-up. When it is necessary to isolate bifunctional compounds from complex sources, columns containing immobilized boronic acid groups have been used and advantage taken of the reversibility of the boronate complexation reaction for attachment and later removal of the desired bifunctional compound.

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CHREV. 132

GAS-LIQUID CHROMATOGRAPHIC RETENTION INDICES OF 296 NON-DRUG SUBSTANCES ON SE-30 OR OV-1 LIKELY TO BE ENCOUNTERED IN TOXICOLOGICAL ANALYSES

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1. INTRODUCTION

Recent advances in gas-liquid chromatography (GLC) have resulted in the widespread availability and use of highly selective detectors which enable toxicologists to detect smaller quantities of drug substances in their extracts than has previously been possible. The use of selective detectors such as the electron capture detector (ECD) for the identification of amenable compounds and the heated alkaline bead thermionic phosphorus/nitrogen detector (PND) for the detection of phosphorus and nitrogen containing compounds was anticipated to remove many of the extraneous peaks frequently observed when non-selective GLC detectors such as the flame ionization detector (FID) were used. The use of these selective detectors has indeed increased the sensitivity of detection of many drug classes, but has exchanged one set of problems for another. Hitherto undetected non-drug substances which interfere in many toxicological analyses are now observed.

One of the major groups of these interfering compounds encountered during

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toxicological analyses is the plasticizers. The term plasticizer as used here encompasses a wide range of compounds, usually esters of alcohols and dibasic or tribasic acids, which are also used in lubricants, coatings, propellants and PVC products etc.¹. These substances have been introduced into biological samples prior to toxicological analyses from containers in which specimens were collected or stored²⁻⁵ and have also been observed to compete with drugs for protein binding sites^{6,7}.

Plasticizers exhibit retention characteristics similar to those of drugs⁸, but are by no means the only interfering compounds found during the GLC of toxicological extracts. Other compounds from plastics and rubber, *e. g.* antioxidants and plastic additives, as well as compounds naturally occurring in biological materials or which are formed as part of a putrefactive process may interfere with an analysis. Pesticides, food additives, flavours, fragrances and some scintillation chemicals are also observed from time to time. We have therefore examined the chromatographic characteristics of these interfering compounds, as well as compounds used as internal standards to allow their quick separation and identification.

The low polarity stationary phases SE-30 or OV-1 have been shown to be the most suitable for toxicological analyses⁹ and are probably the most widely used stationary phases for the routine gas chromatography of drug substances¹⁰⁻¹⁵. Therefore a compilation of retention indices for 296 of these non-drug substances likely to be present in extracts of body fluids and tissues has been made using SE-30 or OV-1 as the stationary phase to update the compilation of data for 480 drugs made by Moffat¹⁶.

2. EXPERIMENTAL

GLC of compounds on OV-1 was performed using a 4 m \times 3 mm I.D. glass column in a Perkin-Elmer F17 gas chromatograph fitted with FID and PND. Helium carrier gas, maintained at 50 ml/min, was split 50:50 after leaving the column enabling chromatograms to be recorded showing the responses from two detectors simultaneously. OV-1 stationary phase (3%, w/w) was coated on Chromosorb W HP (80-100 mesh). Simultaneous detection was achieved using twin amplifiers and a dual pen recorder.

GLC of compounds on SE-30 was performed using a 1.5 m \times 4 mm I.D. glass column packed with 3% (w/w) SE-30 on Chromosorb G HP (80-100 mesh) in a Pye 104 gas chromatograph fitted with ECD and FID. Nitrogen carrier gas was maintained at 60 ml/min and the column effluent split 50:50 as above to each detector.

Even-numbered straight-chain hydrocarbons were used as references for the calculation of retention indices¹⁷. Retention times were measured from the solvent front using either an integrator or a ruler.

A magnetic card programmable calculator was used to perform a least-squares regression analysis of log retention time against retention index (carbon number \times 100). A correlation coefficient of 0.999 was easily obtained. This method was most convenient but readily acceptable results could be obtained using semi-log graph paper. Data was obtained for compounds with retention indices between 695 and 3800. Compounds were eluted by adjusting the oven temperatures so that the retention times of compounds were between 2 and 20 min.

Published data was used to supplement this compilation with values for pesticides. This information, published as relative retention times on DC-200¹⁸ was transformed into retention indices on SE-30 after calibration curves of log relative retention times on DC-200 plotted against retention indices on SE-30 had been constructed for those compounds for which both sets of data were available.

Mass spectral characterisation of some plasticizers exhibiting multiple peaks by GLC were measured using a VG Micromass 16F mass spectrometer linked with a Pye series 104 gas chromatograph.

3. RESULTS AND DISCUSSION

3.1. Compilation of data

The compounds examined during this work have been classified into seven general categories (Table 1) and the abbreviations corresponding to each category have been inserted into the subsequent tables at the appropriate places. Table 2 comprises a list of retention indices for compounds arranged in alphabetical order and the same data, with exception of those giving multiple peaks, are rearranged into ascending order of retention indices in Table 3. The names of the compounds used in this study were those in the *Merck Index*¹⁹ or for the economic poisons the *Nanogen Index*²⁰.

Most of the data in Tables 2 and 3 were generated on the 4-m 3% OV-1 column. Thompson *et al.*¹⁸ published data for 52 pesticides on the dimethylsilicone

TABLE 1

TABLE OF ABBREVIATIONS USED IN THE TABLES OF RETENTION DATA

Classification

A	Antioxidants
D	Putrefactive and endogenous compounds
E	Pesticides (economic poisons)
F	Food additives, flavours and fragrances
I	Internal standards
P	Plasticisers, plastic additives, vulcanising agents
S	Scintillation reagents

Compound abbreviation

BBO	2,5-Di-(4-biphenyloxazole)
BBOT	2,5-Bis(5'- <i>tert.</i> -butylbenzoxazolyl(2'))thiophene
BHC	Benzene hexachloride
CDEC	2-Chloroallyldiethyldithiocarbamic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
DCPA	Dimethyl-2,3,5,6-tetrachloroterephthalate
DDA	2,2-Bis(4-chlorophenyl)acetic acid
DDE	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethylene
DDT	1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane
PBD	2-Phenyl-5-(4-biphenylyl)-1,3,4-oxadiazole
POPOP	1,4-Bis(5-phenyloxazolyl-2)benzene
PPO	2,5-Diphenyloxazole
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
TDE <i>o, p'</i>	1,1-Dichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane
TDE <i>p, p'</i>	1,1-Dichloro-2,2-bis(4-chlorophenyl)ethane

TABLE 2

RETENTION INDICES OF 296 NON-DRUG COMPOUNDS, USING SE-30 OR OV-1 AS THE STATIONARY PHASE, ARRANGED IN ALPHABETICAL ORDER OF COMPOUND NAME

NO = No peak observed; MP = multiple peaks, *i.e.* more than three.

<i>Compound</i>	<i>Retention index</i>	<i>Classification</i>
Acetylcholine	NO	D
Acetyltributyl citrate	2253	P
Acetyltriethyl citrate	1730	P
Adenine	NO	D
Aldrin	1950*	E
Alphanol 610*** (esters of C6-10 linear alcohols)	MP	P
3-Amino-2-dimethylbenzene	1178	I
5-Aminoquinoline	1598	I
Anethole	1284	F
Anilazine	2010	E
Anthracene	1711	I
9,10-Anthracenedicarbonitrile	2288	I
Atrazine	1705**	E
Azinphosmethyl	2430**	E
Azobenzene	1556	I
Barkite B***	MP	P
BBO	3710	S
BBOT	2745	S
	3750§	
Benzophenone	1611	F
Benzyl alcohol	1046	F
Benzyl benzoate	1738	F
α -BHC	1690**	E
β -BHC	1710**	E
δ -BHC	1755**	E
Biphenyl	1389	I
4,4-Bipyridyl dihydrate	1507	I
Bisoflex 1001***	MP	P
1-Bromodecane	1326	I
Bromonaphthalene	1434	I
Bulan***	2310**	E
2-Butoxyethyl oleate	MP	P
Butylated hydroxyanisole	1462	A
Butylated hydroxytoluene	1490	A
Butylbenzyl phthalate	2327	P
Butylbenzyl sebacate	1585	P
	2130	
	2520	
Butyl epoxystearate	MP	P
Butylisodecyl phthalate	1950	P
Butyl oleate	MP	P
Butyl-PBD	3342	S
Butylsextyl phthalate	1940	P
	2235	
Butyl stearate	2157	P
	2362	
Cadaverine	1035	D
Camphor	1137	P
Captan	2000**	E

TABLE 2 (continued)

<i>Compound</i>	<i>Retention index</i>	<i>Classification</i>
Carbazole	1784	I
Carbophenothion	2255**	E
Castor oil	MP	P
CDEC	1685**	E
Chlorobenside	2040	E
Chlordecone	2240**	E
<i>o</i> -Chlorobenzylidenemalonitrile	1516	E
5 α -Cholestane	2852	I
Cholesterol	3008	D
Choline	NO	D
Citral	1272	F
Citroflex A4***	2224	P
Cresylphenyl phosphate	MP	P
Cyclododecanone	1524	E
Cyclohexylisooctyl phthalate	2446	P
	2532	
Cyclohexyltridecyl phthalate	2518	P
2,4-D butyl ester	1840**	E
2,4-D isobutyl ester	1805**	E
2,4-D isopropyl ester	1700**	E
2,4-D methyl ester	1605**	E
DCPA	1960**	E
DDA methyl ester	2085**	E
<i>o</i> , <i>p</i> '-DDE	2070**	E
<i>p</i> , <i>p</i> '-DDE	2130**	E
<i>o</i> , <i>p</i> '-DDT	2220**	E
<i>p</i> , <i>p</i> '-DDT	2290*	E
Demeton-S-methyl	1628	E
Dialkyl adipate 79***	MP	P
Diallyl phthalate	1698	P
Dialphanol phthalate	MP	P
Dialphanol sebacate	MP	P
Diamyl phthalate	2140	P
Diazinon	1760*	E
Dibenzyl phthalate	2690	P
Dibenzyl sebacate	2135	P
<i>m</i> -Dibromobenzene	1197	I
<i>o</i> -Dibromobenzene	1221	I
<i>p</i> -Dibromobenzene	1193	I
<i>p</i> -Dibutoxyethoxyethyl adipate	1285	P
Di-(butoxyethyl) phthalate	2850	P
Di-(butoxyethyl) sebacate	2700	P
Dibutyl adipate	1660	P
Dibutyl maleate	1505	P
Di- <i>n</i> -butyl phthalate	1924	P
Dibutyl sebacate	2137	P
Di- <i>n</i> -butyl terephthalate	2066	P
Dibutyltin dilaurate	NO	P
Dichlone	1760**	E
<i>p</i> -Dichlorobenzene	1000	E
Dicyclohexyl adipate	2282	P
Dicyclohexyl oxalate	1880	P

(Continued on p. 190)

TABLE 2 (*continued*)

<i>Compound</i>	<i>Retention index</i>	<i>Classification</i>
Dicyclohexyl phthalate	2461	P
Dieldrin	2100**	E
Di(ethoxyethyl) adipate	1880	P
Di(ethoxyethyl) phthalate	2135	P
Di(ethoxyethyl) sebacate	2270	P
Diethyl adipate	1349	P
Di(2-ethylhexyl) adipate	2381	P
Di(2-ethylhexyl) isophthalate	2730	P
Di(2-ethylhexyl) phthalate	2507	P
Di(2-ethylhexyl) sebacate	2792	P
Diethyl maleate	1081	P
Diethyl phthalate	1568	P
Diethyl sebacate	1746	P
N,N-Diethyl-N,N-toluamide	1571	E
Diheptyl phthalate	2500	P
9,10-Dihydroanthracene	1662	I
Diisobutyl adipate	1660	P
Diisobutyl phthalate	1863	P
Diisobutyl terephthalate	1972	P
Diisodecyl adipate	2745	P
Diisodecyl phthalate	2511	P
Diisoheptyl phthalate	MP	P
Diisooctyl adipate	MP	P
Diisooctyl epoxystearate	MP	P
Diisooctyl maleate	MP	P
Diisooctyl phthalate	MP	P
Diisooctyl sebacate	MP	P
Dimethoate	1720	E
Dimethoxyethyl phthalate	1980	P
Dimethyl adipate	1223	P
<i>p</i> -Dimethylaminobenzaldehyde	1528	I
Dimethyl isophthalate	1488	P
α,α -Dimethyl- β -methylsuccinamide	1195	I
Dimethyl phthalate	1434	P
Dimethyl POPOP	3618	S
2,4-Dimethyl quinoline	1446	I
2,7-Dimethyl quinoline	1425	I
Dimethyl sebacate	1645	P
Dimethyl terephthalate	1475	P
Dinonyl adipate	2484	P
Dinonyl phthalate	2649	P
Dioctyl adipate	2383	P
Dioctyl phthalate	2519	P
Dioctyl sebacate	2782	P
Di(<i>n</i> -decyl) adipate	2905	P
Diphenyl adipate	2397	P
Diphenyl mercury	1873	P
Diphenyl phthalate	2550	P
Dipropyl adipate	1545	P
Dipropyl phthalate	1743	P
Disextyl maleate	2116	P
Ditridecyl phthalate	NO	P
Dyrene	2010**	E

TABLE 2 (*continued*)

<i>Compound</i>	<i>Retention index</i>	<i>Classification</i>
Endosulfan I	2085**	E
Endosulfan II	2175**	E
Endrin	2165	E
Ethanolamine	780	D
Ethion	2220**	E
Ethylamine	NO	D
Ethylan	2175	E
Ethylbenzoate	1227	F
Ethyl oleate	2175	P
5-Ethyl-5- <i>p</i> -tolyl barbituric acid	2085	I
Eugenol	1368	F
Fluorene	1580	I
Fluorenone	1705	I
Folpet	2015**	E
Glyceryl dibenzoate	2442	P
Glycerol	NO	P
Harman	2000	D
Heptachlor	1890**	E
Heptachlor epoxide	2015**	E
Hexaphenyl benzene	NO	S
Howflex GBP***	1947	P
1-Hydroxychloridene	1955**	E
1-Hydroxyharman	1920	D
	2015	
	2290	
Imidazole	1095	D
Indene	1062	I
Indole	1276	D
Isatin	1712	I
Isobutylcyclohexyl phthalate	1868	P
	2159 [§]	
	2453	
3-Isobutyl-1-methyl xanthine	2150	I
Isobutylsextyl phthalate	MP	P
Isoheptylcyclohexyl phthalate	MP	P
Isooctyl epoxystearate	MP	P
Isooctylisodecyl phthalate	MP	P
Lankroflex 79LP***	MP	P
Lankroflex 79LTM***	MP	P
Limonene	1053	F
Lindane	1757*	E
Linevol 79 phthalate***	MP	P
Linevol 911 phthalate***	MP	P
Linseed oil	MP	P
Liquid paraffin	MP	P
Malathion	1900*	E
2-Mercaptobenzoxazole	NO	P
2-Mercaptobenzimidazole	NO	P
2-Mercaptobenzothiazole	1936	P
Methoxychlor	2410*	E

(*Continued on p. 192*)

TABLE 2 (*continued*)

<i>Compound</i>	<i>Retention index</i>	<i>Classification</i>
Methyl anthranilate	1343	I
Methyl caprylate	1130	I
Methyl decanoate	1305	D
Methyldiphenyl phosphate	MP	P
Methyl <i>p</i> -hydroxybenzoate	1419	A
Methyl linoleate	2100	D
2-Methylnaphthalene	1313	I
Methyl nonanoate	1215	D
Methyl oleate	2086	P
Methyl palmitate	1867	D
5-Methyl-5-phenylhydantoin	1866	I
5(<i>p</i> -Methylphenyl)-5-phenylhydantoin	2457	I
Methyl stearate	2116	P
Mevinphos	1450**	E
Mirex	2470**	E
Monocresyldiphenyl phosphate	MP	P
Morpholine	810	D
Naphthalene	1186	S
1-Naphthonitrile	1489	I
2-Naphthyl acetate	1585	I
Nicotinamide	1475	D
Norharman	2005	D
Octyldecyl adipate	2540	P
	2745	
	2940	
4- <i>tert</i> .-Octyl-2-methyl-cyclohexyl acetate	1611	P
Oleamide	MP	P
Parathion	1935**	E
Parathion methyl	1845	E
Perthane	2175*	E
Phenazine	1703	I
1-Phenylethylamine	1050	D
2-Phenylethylamine	1125	D
Phorate	1675**	E
Piperidine	790	D
Pliabrac A ***	MP	P
Pliabrac 519 ***	MP	P
Pliabrac 521 ***	MP	P
Pliabrac 524 ***	MP	P
Pliabrac 985 ***	MP	P
Pliabrac 987 ***	MP	P
Pliabrac 989 ***	MP	P
Pliabrac 990 ***	MP	P
POPOP	3525	S
PPO	2050	S
Prazepam	2610	I
Prolan	2250**	E
Propylene glycol adipate	MP	P
Propyl- <i>p</i> -hydroxybenzoate	1567	A
Putrescine	930	D
Pyrene	1983	I
Pyribenzamine	1980	I
Pyrrolidine	695	D
Quinoline	1247	I
Ronnel	1880**	E

TABLE 2 (continued)

Compound	Retention index	Classification
Sancticiser 141***	2410 [§]	P
Sancticiser 148***	MP	P
SKF 525A***	2326	I
Simazine	1690**	E
Stigmasterol	3234	I
<i>trans</i> -Stilbene	1755	I
2,4,5-T isopropyl ester	1825**	E
2,4,5-T methyl ester	1740**	E
TDE <i>o,p'</i> isomer	2130	E
TDE <i>p,p'</i> isomer	2200	E
<i>p</i> -Terphenyl	2208	I
Terpineol (mixed isomers)	1127	F
	1170	
	1183	
Tetrachlorvinphos <i>Z</i> isomer	2084	E
Tetradifon	2430**	E
Tetrahydrofurfuryl oleate	2290	P
	2480	
	2660	
Tetraphenylethylene	2478	I
Thianaphthene	1200	I
Thianthrene	1901	P
Triacetin	1282	P
Tributyrin	1552	P
Tri(2-chloroethyl) phosphate	1740	P
Tribenzylamine	2271	I
Tributyl citrate	2150	P
Tributyl phosphate	1690	P
Tricresyl phosphate	2695	P
Triethyl citrate	1655	P
Tri(2-ethylhexyl) phosphate	2463	P
Triethyl phosphate	1109	P
Triflubazam	2244	I
Triisobutyl phosphate	1483	P
Triisopropyl phosphate	1182	P
Trimethyl citrate	1442	P
Trimethyl phosphate	995	P
Trioctyl phosphate	2445	P
Triolein	NO	P
Triphenylamine	2055	I
Triphenyl phosphate	2363	P
Tripropyl phosphate	1372	P
Triptylene	2224	I
Tris(butoxyethyl) phosphate	2363	P
Tris(2,3,-dibromo-propyl) phosphate	NO	P
Tris(2,3,-dichloropropyl) phosphate	2307	P
Tris(isopropylphenyl) phosphate	MP	P
Tritolyl phosphate	MP	P
Trixylenyl phosphate	MP	P
Tryptamine	1750	D
Tyramine	1405	D
Uracil	NO	D
Xanthine	NO	I

* From ref. 16.

** From ref. 18.

*** Trade name.

[§] Major peak.

TABLE 3

RETENTION INDICES OF 253 COMPOUNDS, USING SE-30 OR OV-1 AS THE STATIONARY PHASE, ARRANGED IN ASCENDING ORDER OF RETENTION INDEX

NO = No peak observed; MP = multiple peak, *i.e.* more than three.

<i>Compound</i>	<i>Retention index</i>	<i>Classification</i>
Acetylcholine	NO	D
Adenine	NO	D
Choline	NO	D
Dibutyltin dilaurate	NO	P
Ditridecyl phthalate	NO	P
Ethylamine	NO	D
Glycerol	NO	P
Hexaphenyl benzene	NO	S
2-Mercaptobenzimidazole	NO	P
2-Mercaptobenzoxazole	NO	P
Triolein	NO	P
Tris(2,3-dibromopropyl) phosphate	NO	P
Uracil	NO	D
Xanthine	NO	I
Pyrrolidine	695	D
Ethanolamine	780	D
Piperidine	790	D
Morpholine	810	D
Putrescine	930	D
Trimethyl phosphate	995	P
<i>p</i> -Dichlorobenzene	1000	E
Cadaverine	1035	D
Benzyl alcohol	1046	F
1-Phenylethylamine	1050	D
Limonene	1053	F
Indene	1062	I
Diethyl maleate	1081	P
Imidazole	1095	D
Triethyl phosphate	1109	P
2-Phenylethylamine	1125	D
Terpineol (mixed isomers)	1127	F
	1170	
	1183	
Methyl caprylate	1130	I
Camphor	1137	P
3-Amino-2-dimethylbenzene	1178	I
Triisopropyl phosphate	1182	P
Naphthalene	1186	S
<i>p</i> -Dibromobenzene	1193	I
α,α -Dimethyl- β -methyl succinamide	1195	I
<i>m</i> -Dibromobenzene	1197	I
Thianaphthene	1200	I
Methyl nonanoate	1215	D
<i>o</i> -Dibromobenzene	1221	I
Dimethyl adipate	1223	P
Ethylbenzoate	1227	F
Quinoline	1247	I
Citral	1272	F
Indole	1276	D
Triacetin	1282	P

TABLE 3 (continued)

Compound	Retention index	Classification
Anethole	1284	F
<i>p</i> -Dibutoxyethoxyethyl adipate	1285	P
Methyl decanoate	1305	D
2-Methylnaphthalene	1313	I
1-Bromodecane	1326	I
Methyl anthranilate	1343	I
Diethyl adipate	1349	P
Eugenol	1368	F
Tripropyl phosphate	1372	P
Biphenyl	1389	I
Tyramine	1405	D
Methyl <i>p</i> -hydroxybenzoate	1419	A
2,7-Dimethylquinoline	1425	I
Bromonaphthalene	1434	I
Dimethyl phthalate	1434	P
Trimethyl citrate	1442	P
2,4-Dimethylquinoline	1446	I
Mevinphos	1450**	E
Butylated hydroxyanisole	1462	A
Dimethyl terephthalate	1475	P
Nicotinamide	1475	D
Triisobutyl phosphate	1483	P
Dimethyl isophthalate	1488	P
1-Naphthonitrile	1489	I
Butylated hydroxytoluene	1490	A
Dibutyl maleate	1505	P
4,4-Bipyridyl dihydrate	1507	I
<i>o</i> -Chlorobenzylidenemalonitrile	1516	E
Cyclododecanone	1524	E
<i>p</i> -Dimethylaminobenzaldehyde	1528	I
Dipropyl adipate	1545	P
Tributylin	1552	P
Azobenzene	1556	I
Propyl- <i>p</i> -hydroxybenzoate	1567	A
Diethyl phthalate	1568	P
N,N-Diethyl-N,N-toluamide	1571	E
Fluorene	1580	I
2-Naphthyl acetate	1585	P
Butylbenzyl sebacate	1585	P
	2130	
	2520	
5-Aminoquinoline	1598	I
2,4-D methyl ester	1605**	E
Benzophenone	1611	F
4- <i>tert</i> .-Octyl-2-methyl-cyclohexyl acetate	1611	P
Demeton-S-methyl	1628	E
Dimethyl sebacate	1645	P
Triethyl citrate	1655	P
Dibutyl adipate	1660	P
Diisobutyl adipate	1660	P
9,10-Dihydroanthracene	1662	I
Phorate	1675**	E

(Continued on p. 196)

TABLE 3 (continued)

Compound	Retention index	Classification
CDEC	1685**	E
α -BHC	1690**	E
Tributyl phosphate	1690	P
Simazine	1690**	E
Diallyl phthalate	1698	P
2,4-D isopropyl ester	1700**	E
Phenazine	1703	I
Atrazine	1705**	E
Fluorenone	1705	I
β -BHC	1710**	E
Anthracene	1711	I
Isatin	1712	I
Dimethoate	1720**	E
Acetyltriethyl citrate	1730	P
Benzyl benzoate	1738	F
2,4,5-T methyl ester	1740**	E
Tri(2-chloroethyl) phosphate	1740	P
Dipropyl phthalate	1743	P
Diethyl sebacate	1746	P
Tryptamine	1750	D
δ -BHC	1755**	E
<i>trans</i> -Stilbene	1755	I
Lindane	1757*	E
Diazinon	1760*	E
Dichlone	1760	E
Carbazole	1784	I
2,4-D isobutyl ester	1805**	I
2,4,5-T isopropyl ester	1825**	E
2,4-D butyl ester	1840**	E
Parathion methyl	1845	E
Diisobutyl phthalate	1863	P
5-Methyl-5-phenylhydantoin	1866	I
Methyl palmitate	1867	D
Isobutylcyclohexyl phthalate	1868	P
	2159 [§]	
	2453	
Diphenyl mercury	1873	P
Dicyclohexyl oxalate	1880	P
Di(ethoxyethyl) adipate	1880	P
Ronnel	1880**	E
Heptachlor	1890**	E
Malathion	1900*	E
Thianthrene	1901	I
1-Hydroxyharman	1920	D
	2015	
	2290	
Di- <i>n</i> -butyl phthalate	1924	P
Parathion	1935**	E
2-Mercaptobenzothiazole	1936	P
Butylsextyl phthalate	1940	P
	2235	
Howflex GBP***	1947	P
Aldrin	1950*	E
Butylisodecyl phthalate	1950	P
1-Hydroxychlorodene	1955**	E
DCPA	1960**	E
Diisobutyl terephthalate	1972	P

TABLE 3 (continued)

Compound	Retention index	Classification
Pyribenzamine	1980	I
Pyrene	1983	I
Captan	2000**	E
Harman	2000	D
Norharman	2005	D
Anilazine	2010	E
Dyrene	2010**	E
Folpet	2015**	E
Heptachlor epoxide	2015**	E
Chlorobenside	2040**	E
PPO	2050	S
Triphenylamine	2055	I
Di- <i>n</i> -butyl terephthalate	2066	P
<i>o</i> , <i>p</i> -DDE	2070**	E
Tetrachlorvinphos <i>Z</i> isomer	2084	E
DDA methyl ester	2085**	E
Endosulfan I	2085**	E
5-Ethyl-5- <i>p</i> -tolyl barbituric acid	2085	I
Methyl oleate	2086	P
Dieldrin	2100*	E
Methyl linoleate	2100	D
Disextyl maleate	2116	P
Methyl stearate	2116	P
<i>p</i> , <i>p'</i> -DDE	2130**	E
TDE <i>o</i> , <i>p'</i> isomer	2130	E
Di(ethoxyethyl) phthalate	2135	P
Dibenzyl sebacate	2135	P
Dibutyl sebacate	2137	P
Diamyl phthalate	2140	P
3-Isobutyl-1-methyl xanthine	2150	I
Tributyl citrate	2150	P
Butyl stearate	2157	P
	2362	
Endrin	2165**	E
Endosulfan II	2175**	E
Ethylan	2175	E
Ethyl oleate	2175	P
Perthane	2175*	E
TDE <i>p</i> , <i>p'</i> isomer	2200	E
<i>p</i> -Terphenyl	2208	I
<i>o</i> , <i>p'</i> -DDT	2220**	E
Ethion	2220**	E
Citroflex A4***	2224	P
Triptylene	2224	I
Chlordecone	2240**	E
Triflubazam	2244	I
Prolan	2250**	E
Acetyltributyl citrate	2253	P
Carbophenothion	2255**	E
Di(ethoxyethyl) sebacate	2270	P
Tribenzylamine	2271	I
Dicyclohexyl adipate	2282	P
9,10-Anthracenedicarbonitrile	2288	I
<i>p</i> , <i>p'</i> -DDT	2290*	E
Tetrahydrofurfuryl oleate	2290	P
	2480	
	2660	

TABLE 3 (*continued*)

<i>Compound</i>	<i>Retention index</i>	<i>Classification</i>
Tris(2,3-dichloropropyl)phosphate	2307	P
Bulan***	2310**	E
SKF 525A***	2326	I
Butylbenzyl phthalate	2327	P
Triphenyl phosphate	2363	P
Tris(butoxyethyl) phosphate	2363	P
Di(2-ethylhexyl) adipate	2381	P
Diocetyl adipate	2383	P
Diphenyl adipate	2397	P
Sancticiser 141***	2410§	P
Methoxychlor	2410*	E
Azinphosmethyl	2430**	E
Tetradifon	2430**	E
Glyceryl dibenzoate	2442	P
Trioctyl phosphate	2445	P
Cyclohexylisooctyl phthalate	2446	P
	2532	
5(<i>p</i> -Methylphenyl)-5-phenylhydantoin	2457	I
Dicyclohexyl phthalate	2461	P
Tri(2-ethylhexyl) phosphate	2463	P
Mirex	2470**	E
Tetraphenylethylene	2478	I
Dinonyl adipate	2484	P
Diheptyl phthalate	2500	P
Di(2-ethylhexyl) phthalate	2507	P
Diisodecyl phthalate	2511	P
Cyclohexyltridecyl phthalate	2518	P
Diocetyl phthalate	2519	P
Octyldecyl adipate	2540	P
	2745	
	2940	
Diphenyl phthalate	2550	P
Prazepam	2610	I
Dinonyl phthalate	2649	P
Dibenzyl phthalate	2690	P
Tricresyl phosphate	2695	P
Di(butoxyethyl)sebacate	2700	P
Di(2-ethylhexyl) isophthalate	2730	P
Di(isodecyl) adipate	2745	P
BBOT	2745	S
	3750§	
Diocetyl sebacate	2782	P
Di(2-ethylhexyl) sebacate	2792	P
Di(butoxyethyl) phthalate	2850	P
5 α -Cholestane	2852	I
Di(<i>n</i> -decyl) adipate	2905	P
Cholesterol	3008	D
Stigmasterol	3234	I
Butyl PBD	3342	S
POPOP	3525	S
Dimethyl POPOP	3618	S
BBO	3710	S

* From ref. 16.

** From ref. 18.

*** Trade name.

§ Major peak.

phase DC-200 expressed as retention ratios relative to aldrin. Seven data points from this collection were plotted as log retention ratios against known retention indices¹⁶. The regression obtained was:

$$\text{Retention index on SE-30} = 706 (\log \text{ retention ratio}) + 1955$$

with a correlation coefficient of 0.9993. The rest of the data were then transformed into retention indices on SE-30 using the equation above and included in Tables 2 and 3. The transformation of retention times, retention ratios and retention indices using the same, or closely related, stationary phases is therefore an easy way to compile standardised data without the need to rechromatograph every substance.

The use of retention indices for standardisation in GLC has enabled compilations of data to be made for use in toxicological analyses^{13,14,16,21}. For these collections to be of the greatest use, it is imperative that the factors affecting the reproducibility of retention indices are recognised and precautions taken to minimise the errors of measurement. The choice of support is an important feature²² and in general it should be inactivated as thoroughly as possible. The effects of the residual active sites may be reduced by using a high-polarity phase or by using a sufficiently high loading of stationary phase^{22,23}. High-polarity phases unfortunately give poorer reproducibility than low-polarity phases⁹ so that a good compromise is between 2 and 5% of a low-polarity phase. The temperature is another very important feature and it has been shown by several authors that the retention index is dependent on the temperature of the column^{21,24,25} so that the measurement of very short or very long retention times should be avoided. Thus, to obtain reproducible results the conditions used for chromatography should not be too far removed from those used by the workers who compiled the data. The sample size is important²⁶ since longer retention times will be observed for polar materials as the quantity chromatographed is decreased.

In order to compensate for different conditions in different laboratories (e.g. support, different phase loadings, temperature, etc.) Moffat¹⁶ suggested that an error factor of ± 50 retention index units would be an acceptable limit of reproducibility for measurements based on a standard deviation of 18 retention index units. To measure the reproducibility of results between our two laboratories, 17 compounds were randomly selected from the various chemical classes of substances in this collection and retention indices of these compounds were measured independently in each laboratory. Despite the fact that a 4 m \times 3 mm I.D. column of 3% (w/w) OV-1 on Chromosorb W HP was used in one laboratory and a 1.5 m \times 4 mm I.D. column of 3% (w/w) SE-30 on Chromosorb G HP was used in the second laboratory, the mean interlaboratory difference between indices for the 17 compounds was ± 13 retention index units, with only one outside the ± 50 limit (difference of 55 units). This supports the claims that the use of retention index measurements provides a good basis for peak identification procedures.

For a more rigorous identification of an ester, it is possible to use the method suggested by Krishen¹. This involves the hydrolysis of the ester, and subsequent chromatography of the alcohol and the acid after it has been methylated.

A total of 14 compounds did not elute under the conditions used in this work. However, the majority of compounds eluted as single peaks, although a few excep-

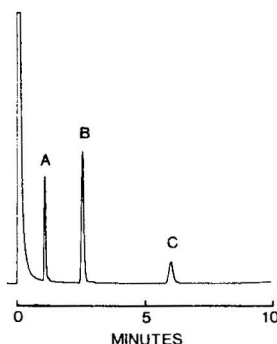


Fig. 1. Chromatogram of a commercial sample of isobutylcyclohexyl phthalate using FID. A, diisobutyl phthalate; B, isobutylcyclohexyl phthalate; C, dicyclohexyl phthalate.

tions were noted. The retention indices of the 8 compounds giving up to three significant peaks have been incorporated into Tables 2 and 3. In most cases, where more than one major peak was observed, the identity of the extra peaks reflected the mode of synthesis of the supposedly primary compound. For example, three peaks were observed in the chromatogram of isobutylcyclohexyl phthalate (Fig. 1) which were identified by mass spectrometry as diisobutyl phthalate, isobutylcyclohexyl phthalate and dicyclohexyl phthalate. Other examples are given in Table 4. Some compounds (a total of 44) revealed even more complex chromatograms. In cases where more than three significant peaks were observed, the compound was listed in Table 2 as having multiple peaks and omitted from Table 3. A list of compounds chromatographing as multiple peaks is presented in Table 5 and an example is shown in Fig. 2. The complex nature of some of these substances may be indicated by a "noisy baseline" rather than by distinct peaks.

TABLE 4

IDENTIFICATION OF SOME COMPOUNDS EXHIBITING MULTIPLE PEAKS BY GAS-LIQUID CHROMATOGRAPHY

<i>Compound</i>	<i>Peak identification (by mass spectrometry)</i>
Octyldecyl adipate	Octyl adipate Dioctyl adipate Octyldecyl adipate
Cresylphenyl phosphate	Diphenylcresyl phosphate Dicresylphenyl phosphate Tricresyl phosphate
Isobutylcyclohexyl phthalate	Diisobutyl phthalate Dicyclohexyl phthalate Isobutylcyclohexyl phthalate
Butylbenzyl sebacate	Dibutyl sebacate Butylbenzyl sebacate Dibenzyl sebacate
Isooctylisodecyl phthalate	Diisooctyl phthalate Isooctylisodecyl phthalate Diisodecyl phthalate

TABLE 5

ALPHABETICAL LIST OF COMPOUNDS ELUTING AS MULTIPLE PEAKS (*i.e.* MORE THAN THREE)

<i>Compound</i>	<i>Classification</i>
Alphanol 610*	P
Barkite B	P
Bisoflex 1001*	P
2-Butoxyethyl oleate	P
Butyl epoxystearate	P
Butyl oleate	P
Castor oil	P
Cresylphenyl phosphate	P
Dialkyl adipate 79*	P
Dialphanol phthalate	P
Dialphanol sebacate	P
Diisoheptyl phthalate	P
Diisooctyl phthalate	P
Diisooctyl adipate	P
Diisooctyl epoxystearate	P
Diisooctyl maleate	P
Diisooctyl phthalate	P
Diisooctyl sebacate	P
Isobutylsextyl phthalate	P
Isoheptylcyclohexyl phthalate	P
Isooctyl epoxystearate	P
Isooctylisodecyl phthalate	P
Lankroflex 79LP*	P
Lankroflex 79LTM*	P
Linevol 79 phthalate*	P
Linevol 911 phthalate*	P
Linseed oil	P
Liquid paraffin	P
Methyldiphenyl phosphate	P
Monocresyldiphenyl phosphate	P
Oleamide	P
Pliabrac A*	P
Pliabrac 519*	P
Pliabrac 521*	P
Pliabrac 524*	P
Pliabrac 985*	P
Pliabrac 987*	P
Pliabrac 989*	P
Pliabrac 990*	P
Propylene glycol adipate	P
Sancticiser 148*	P
Tris(isopropylphenyl) phosphate	P
Tritolyl phosphate	P
Trixylenyl phosphate	P

* Trade name.

3.2. Examples of interferences by plasticizers

Plasticizers and other non-drug substances may enter biological samples from a variety of sources or may even be added inadvertently during analysis. For example, in a vertical injection system particles of a septum may be dislodged on to the top

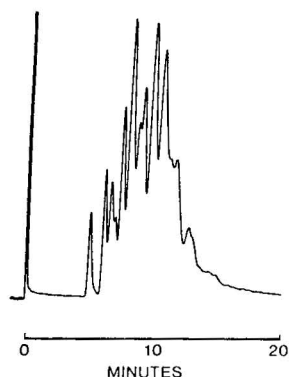


Fig. 2. Chromatogram of a commercial sample of tritolyl phosphate using PND in phosphorus mode.

of the column and if the septum contains a plasticizer, peaks for this compound may be observed. An illustration of the presence of a plasticizer in a GLC septum with similar chromatographic properties to tris(isopropylphenyl) phosphate is shown in Fig. 3.

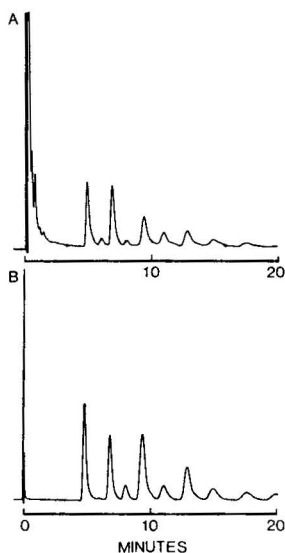


Fig. 3. Chromatograms obtained using PND in phosphorus mode of A, an acetone extract of a GLC septum; B, tris(isopropylphenyl) phosphate.

In addition to the retention index data, the properties of substituents in the molecules may be of aid in the identification of unknown peaks since in many instances the response ratio of compounds between different selective detectors can be characteristic²⁷. The FID response is dependent on the number of carbon atoms in a molecule and is quite predictable. However, the ECD response for different compounds varies widely and is dependent on the electron deficient part of the compound, and is quite difficult to predict. The PND response for a compound depends to some extent on

the number of nitrogen or phosphorus atoms in a molecule but also depends on their environment. It follows that by using the FID as a reference we can measure the ECD or PND response relative to it, thus adding another variable to measure in addition to the retention index.

The ECD is very sensitive to phthalate esters. For example, Fig. 4 illustrates the presence of di(2-ethylhexyl) phthalate (DEHP) as an impurity in a blood extract from a patient who had received a transfusion of blood which had been stored in a plastic bag. The ECD/FID response ratio was of valuable assistance in the differentiation between plasticizer peaks and the compounds of interest (benzodiazepines) since the ECD/FID ratio for DEHP differs considerably from that of benzodiazepines.

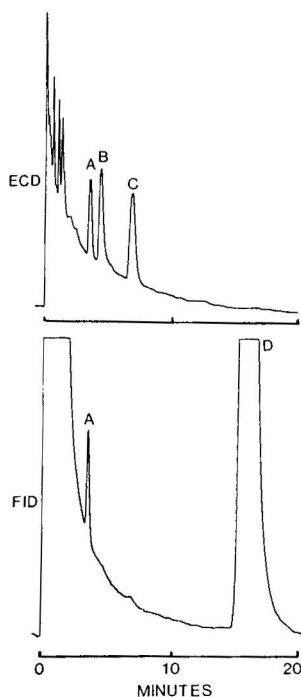


Fig. 4. Chromatograms, using both FID and ECD, of an extract of a blood sample from a patient taking flurazepam and who had had a blood transfusion. A, di(2-ethylhexyl) phthalate from the poly(vinylchloride) transfusion bag; B, desalkylflurazepam (a metabolite of flurazepam); C, prazepam (internal standard); D, cholesterol.

The heated bead PND may be made very highly selective for phosphorus containing compounds, but will retain its sensitivity to phosphorus even when optimised for its nitrogen response. Hence, by running a chromatogram with the PND optimised for phosphorus and then running the chromatogram with the detector optimised for nitrogen it may be deduced if a compound contains nitrogen or phosphorus. In addition, if a dual-detector system (PND/FID) is used, the greater sensitivity of the PND to phosphorus containing compounds compared with nitrogen

containing compounds can be recognised using the FID response as a reference. Fig. 5 illustrates the use of the PND/FID response ratios to reveal the phosphate impurities originating from filter paper present in a blood extract containing barbiturates (which contain nitrogen).

Tables 2 and 3 contain retention data for compounds that have been used as internal standards for the quantitative analyses of drugs. Because of the insidious nature of plasticizers, as shown by the above examples, it is obvious that their use as internal standards may lead to errors.

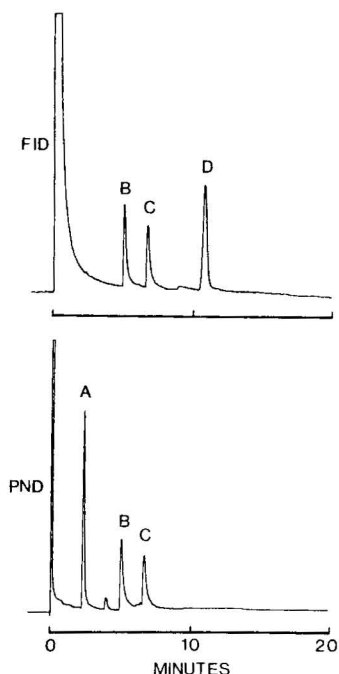


Fig. 5. Chromatograms using FID and PND in nitrogen mode, with attenuations adjusted to give roughly equal sized peaks for the barbiturates on both detectors, of an extract of a blood sample containing barbiturates. The contamination from triisobutyl phosphate introduced from filter paper is apparent. A, triisobutyl phosphate; B, amylobarbitone; C, quinalbarbitone; D, $C_{19}H_{40}$ (retention index marker).

4. CONCLUSIONS

Plasticizers and other non-drug substances may find their way into analytical extracts via a number of different routes. Hence the presence of these impurities may provide analytical complications to the unsuspecting analyst. The retention indices of 296 non-drug substances have been measured on SE-30 or OV-1 to supplement collections of previously published data on these non-polar stationary phases. The use of selective GLC detector response ratios may, in many instances, be of value in differentiating between drug and non-drug substances in biological extracts. The use of plasticizers as internal standards for quantitative analyses is to be discouraged.

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6. SUMMARY

The advent of the widespread use of selective detectors (electron capture detector, phosphorus/nitrogen detector) for gas-liquid chromatography used in toxicological analyses has revealed the presence of hitherto unseen interfering materials. These substances may be conveniently grouped into (1), anti-oxidants; (2), putrefactive and endogenous compounds; (3), pesticides; (4), food additives, flavours and fragrances; (5) plasticisers, plastic additives and vulcanising agents and (6), scintillation reagents. To facilitate the identification of these materials, retention indices on the dimethyl silicone phases SE-30 or OV-1 have been compiled by the two laboratories to include 296 such compounds. Most gave single peaks, but some gave complex patterns indicating that they were mixtures of compounds. Of the 296 compounds, 14 did not give observable peaks, 8 gave 2 or 3 peaks and 44 gave more than 3 peaks. To determine the interlaboratory difference between retention index measurements, 17 compounds were chromatographed by both laboratories: the mean difference was ± 13 retention index units with only one greater than ± 50 retention index units. Examples of how these materials may be encountered during toxicological analyses are given. Data are also presented on compounds which have been used as internal standards.

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CHREV. 129

THE ROLE OF CHROMATOGRAPHY IN CIBA-GEIGY*

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In its present form Ciba-Geigy is only 9 years old, having been founded as a result of the merger of Geigy and Ciba in 1970. However, notwithstanding its apparent youth, the company has a long tradition: Geigy was founded over 200 years ago and Ciba grew out of a silk dyeworks about a century ago. Both the "Parent Company" and the Headquarters of the group are in Basle, and a great part of the research work is performed there. Six production plants in Switzerland or just across its borders form the "parent works", but the majority of the production plants, in the so-called "Group Companies" are spread all over the world.

The products manufactured by Ciba-Geigy can be divided into two groups:

(i) *Industrial products*: dyes for textiles, textile chemicals, pigments and high-grade synthetic materials including preservatives, photographic materials and household products;

(ii) *Biologically active products*: pharmaceuticals, veterinary medicines and plant-protection agents.

The Group has a turnover of over US\$ 5000 million and employs about 75,000 people. The staff working in Switzerland in the Headquarters and the different production plants number nearly 20,000, of whom 2000 are university-trained and 900 are technicians. Apart from Ciba-Geigy there are two other large chemical companies in Basle, Hoffmann-La Roche and Sandoz, and there is a continuing exchange of experience in analytical problems between these companies. The analytical personnel working in the Parent Company, including the Swiss production plants, are employed in three different organisations: Central Analytics, Works Analytics and Divisional Analytical Groups.

Central Analytics (Fig. 1), which is a subdivision of the Central Research Department, consists of product-orientated and method-orientated analytical sections. The product-orientated sections are so organized that each of the Company's four manufacturing divisions, pharmaceuticals, agro-chemicals, dyestuffs and plastics, has its own analytical staff who are particularly familiar with the products and problems of the division concerned. The method-orientated section consists of a group devoted to elemental analysis and a separations group, which is subdivided into units carrying out gas chromatography (GC), liquid chromatography (LC), thin-layer chromatography (TLC) and special tasks.

* Presented at the 14th International Symposium on Advances in Chromatography, Lausanne, September 24–28, 1979.

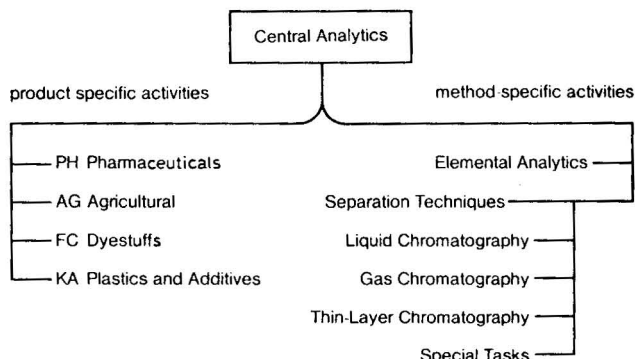


Fig. 1. Organization diagram of Central Analytics.

In addition, each of the Swiss production plants has its own analytical group, called *Works Analytics*. Their main task is to give analytical support to the production personnel. These analytical groups are not controlled by Central Analytics: their work is carried out independently, although there is close collaboration between them and Central Analytics.

All of the other groups engaged in analysis, but not belonging to Central Analytics or Works Analytics, are combined under the heading *Divisional Analytical Groups*. These are mostly special departments with a specific divisional clientele who aim to solve particular problems arising from, e.g., divisional-orientated research, residue analysis, pharmacology or ecology. They are also engaged in in-process control or application tests. Although these groups are outside Central Analytics, the latter provide them with technical advice on methodology.

About 1300 people are engaged in analytical problem solving within these three analytical branches of the Parent Company.

The Separation Group of Central Analytics is engaged almost exclusively in chromatography. Apart from this, chromatographic methods are used extensively by many other analytical groups to find solutions to their problems.

When introducing a new chromatographic method we always proceed as follows:

Phase 1. The technique is introduced in the Separation Group of Central Analytics and subsequently applied to problems of other groups. During this phase know-how is gained, and considerable effort is devoted to instrument development.

Phase 2. The know-how is passed on to other groups, the personnel become familiar with the equipment and the method is appraised. This is the phase when the instruments are adapted specifically for their intended use.

Phase 3. The technique is widely applied in all analytical groups. Central Analytics still work on special problems only during this last phase, but continue to provide back-up service. In Ciba-Geigy, TLC and, in most instances GC, reached this third phase a long time ago, and at present LC is passing from phase 2 to phase 3. Isotachopheresis is a method that seems very promising but has not yet been developed beyond phase 1.

However, there are techniques that are unlikely to be generally passed on from Central Analytics to other analytical groups. These include GC-mass spectrometry

(MS), because of the elaborate instrumentation and special knowledge required, and other combined methods, such as GC-TLC, GC-TEA (thermal energy analyser) and LC-TEA.

Although GC has reached phase 3, Central Analytics itself still deals with many GC problems. There are various reasons for this: the person requesting the analysis may be insufficiently equipped with regard to know-how or instrumentation; a long sample run, permitting computerization, may be involved; or complex problems may arise, requiring specialist knowledge.

In order to solve problems (Fig. 2), the analyst has to find answers to the questions about the *quality*, *quantity* and *behaviour* of the sample under investigation.

The question concerning *qualitative* aspects, *i.e.*, the composition of the sample, is usually tackled first. Indeed, analytical chemistry had its origins in such questions. Whereas in the recent past elemental analysis provided the only indication of the composition of a sample, we now have to concern ourselves with the manner in which the elements are present, *e.g.*, the chemical or physical form in which they are present. Such questions have now to be answered even when only trace elements are involved, *e.g.*, we must differentiate between metallic mercury, its salts and its organic compounds.

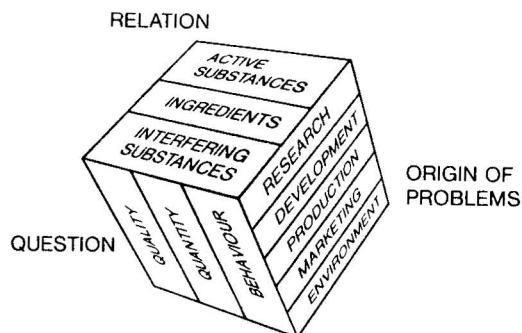


Fig. 2. Analytical problems.

The determination of *quantity* is one of the main tasks of the analyst. The chemical industry sells primarily not substances but effects, and these effects are correlated to the amount of active substance. Here we have a wide spectrum, depending on whether we have to deal with a pure, active substance with an assay of nearly 100%, with a formulated preparation, *e.g.*, with hormones containing 0.1 mg of active substance per tablet, or with residues of pesticides on plants. In the last two instances the matrix in which the active substance is contained is of decisive importance. The art of the analyst consists in completely separating the substance he is seeking from the carrier substance, which is present in a large excess.

When determining the quantity, we have first to decide, as a matter of principle, whether high accuracy or high specificity is desired. The specificity can, for instance, be ensured by a separate identity test.

The crucial points in the investigation of *behaviour* are the determination of reaction kinetics, the stability of active substances, and, to an ever-increasing extent, the decomposition behaviour as it relates to ecological effects. Probably the most

important task of the analyst, involving the elucidation of the decomposition behaviour of such materials (e.g., in the determination of chemicals in sewage), lies in the quantitative separation of trace amounts of contaminating material. Here, specificity is usually more important than accuracy. The equilibrium distribution in a biological system or the behaviour of a substance in a changing environment provide real challenges for the modern analyst.

These three criteria, quality, quantity and behaviour, apply to three classes of material: active substances, additional ingredients and interfering substances.

By *active substances* we mean that part of a product that produces the effect desired by the customer, whether in a biologically active or an industrial product.

Because, of course, there are no chemically pure substances in the absolute sense of the term, we have to be informed about the types and amounts of any *additional ingredients*. By these we mean inert substances, either produced during manufacture or added intentionally as part of the formulation.

Interfering substances are all those substances which impair the intended effect, biologically or technically, and are, therefore, undesirable. The analyst has to have these substances especially under control. Here it is extremely important that the method employed is specific, particularly when trace amounts are involved.

The analytical groups have to concern themselves with problems passed on to them from five different areas: Research, Development, Production, Marketing and Environment.

In the *Research* area, analytical support is provided to synthetic chemists. The analytical work involved is generally undertaken by divisional analysts, who are more familiar with the problems specific to the product. Analytics also determines the constitution of the product by elemental analysis, functional group determinations or measurement of physical parameters.

The work sent from the *Development* area entails the determination of active substance contents and the qualitative and quantitative determination of interfering substances which can be removed at this stage. The determination of the stability of active substances and formulated products is also required, and here the analyst employs the most specific methods available.

The support given by Analytics is most versatile in the area of *Production*. The analytical activities for process control and process guidance must take place as close as possible to the place of production. Usually determinations of active substance content are needed, as are to some extent those of interfering substances. In automatic processes the extent of analytical control may often be reduced without loss of product quality. Here the emphasis lies primarily on speed and economy, provided the method gives informative results, and accuracy and specificity take second place.

Marketing specialists are interested in keeping the price of products competitive while maintaining the quality level promised to the customer or expected by him—as already mentioned, the customer basically does not buy the product but its effect. The quality of design must meet the customer's range of application and the quality of production must meet the quality of design.

The specifications and methods of analysis serve this purpose. Both are prepared mainly by the Parent Company. For certain categories of products they are binding, worldwide, upon all members of the Company Group. From this obligation it follows that the effort involved in changing analytical methods may become con-

siderable. Minor improvement in procedure or equipment employed do not justify this in every instance. Also, if analytical methods demand new instrumental techniques, considerable capital investments may be involved as the analytical departments concerned have to comply. Therefore, a new technique must have decisive advantages in order to be worthwhile.

Problems from customers, passed on by Marketing to Analytics, whether they concern complaints about the quality of the product or requests for analytical support in the application of the product, can only be solved if the analyst has a sound basic knowledge and is flexible in his work.

The last area from which tasks come to Analytics is that of *Environment*. Environmental requirements have always had to be considered, but during the last few years the demands have grown in importance. Firstly, there are official regulations, aimed at ensuring the safety of people involved in production, handling and applications of products or in the protection of the environment. The progress made in this respect is considerable, but so is the danger of over-regulation. The desired aim can best be met by open collaboration between industry and authorities. Here again, the analyst has to make a contribution by suggesting reasonable limits.

Now, what part does chromatography play in the overall analytical effort? For the determination of the content of active substances, additional ingredients or interfering substances, it is usually the method of choice. This is mainly due to the fairly high selectivity attained as a result of good separation, but also to the versatility of the equipment. Generally, it is not extreme accuracy that is aimed at in content determination. A product is usually better characterized by a specific medium-precision determination of content and a quantitative determination of all the additional substances. This combination of separation and quantitative determination, characteristic of chromatographic methods, often provides considerably more information than other methods.

In trace determinations, specificity is usually much more important than accuracy. It will be remembered only too well how it was alleged that the whole world had been contaminated by DDT. Later it was discovered that the values found were not all due to DDT, but partly to polychlorinated biphenyls. Without an exact knowledge of the type of traces we are seeking, we cannot effectively control them.

Therefore, for many reasons, chromatography is the preferred method for the determination of content and purity. This is true for research projects, for products in the development stage, for raw materials, auxiliary substances, intermediate and finished products, and even more so when working on marketing and environmental problems. Wet chemistry still has its place, but it has become far less important than chromatographic methods.

The number of gas chromatographs (300) and liquid chromatographs (nearly 100) in operation in the three analytical organisations in the Parent Company illustrate the above point. About 10% of these instruments are provided with automatic injection and control, so that they can operate continuously. Approximately 200,000 gas chromatograms and 300,000 liquid chromatograms per annum are produced.

Gas chromatography is mostly performed using packed columns, although capillary columns are gaining in importance. Its prerequisites are reliable methods of quantitative injection, with or without sample splitting, and increased knowledge of

column preparation. For packed columns, four stationary phases (OV-17, OV-101, OV-225, Carbowax 20M), based on the work of McReynolds, are used preferentially when developing a separation method. With these four stationary phases, 80 % of the separation problems can be solved. We are thus able to avoid a great deal of unnecessary work, as the numerous other company laboratories do not have to adapt the methods developed by us to their conditions.

Liquid chromatography has grown rapidly in importance, particularly because polar substances can be injected as such, without elaborate derivatization steps, which are not always without problems, and the separation time is shorter than in GC. On the other hand, method development requires a greater effort, until a generally applicable method is found. It is of great importance that the effort required for the adaptation of methods when changing an instrument should be as small as possible. The reversed-phase method has proved helpful. Further, by applying ion-pair distribution to LC we have opened up further interesting possibilities.

Thin-layer chromatography is of particular use in quality testing. Here developments are still taking place, e.g., in the field of better separating chambers, carrier materials, separation systems and detection methods. We consider that the smaller effort needed for the development of an analytical method is an advantage over LC, but the poorer precision in quantitative determinations is a disadvantage. We are also interested in using TLC for preparative separations of additional substances in the milligram range.

We employ the following *Special Methods* of chromatography:

(a) GC-MS, where advantage can be taken of the high specificity of the detector;

(b) GC-TEA and LC-TEA, where the thermal energy analyser is used specifically to determine N-nitrosamines;

(c) preparative and micro-preparative isolation techniques for spectroscopic structure elucidation; here we should mention especially the micro-transfer techniques TLC-MS, TLC-infrared spectroscopy and TLC-nuclear magnetic resonance spectroscopy;

(d) isotachopheresis.

We envisage new possibilities for using further combinations of chromatographic methods, such as LC-MS, and also on-line combinations of derivatization reactions with chromatography.

What do we expect from chromatographic instruments? Briefly, they must be economical to buy and to service, practical and durable in use.

For purchasing, the following calculation can be made. The time an instrument is in use during its life amounts to 10,000–15,000 h. If we assume an average requirement of 1 h to draw a chromatogram and an average operational time of 60 %, then 6000–10,000 determinations can be made with one instrument. At a price of SFr. 30,000–60,000 per instrument, including accessories, this amounts to SFr. 3–10 per determination, which is acceptable.

Things become more critical when an instrument has a high rate of breakdown and more so when the service offered by the manufacturer's representative is inadequate with regard to time or quality. Here we must mention that Central Analytics has been given the task by the various users of chromatographs in the company of evaluating new instruments and gathering experience with existing instruments. As

in other areas, there is an exchange of information with the other companies in this respect.

By "practical" we mean that potential problems can be solved with the desired accuracy and within an acceptable time. For this it is essential, among other things, that alterations which sometimes prove necessary can be made to the instrument with little effort.

What we do not want is a large number of gadgets on the instruments, for instance those that originated from the euphoria about the versatility of microprocessors. They are not of much use, and they impair the desired flexibility in the employment of personnel to tend them. It is the same story with so-called new developments that appear at short intervals, which only concern trivialities. These make the exchange of instruments or parts more difficult. It goes without saying that we expect the instruments to be of high quality and durability.

Finally, some hints and suggestions from the users to the manufacturers may be useful here.

Given equal performance, the users prefer simpler instruments and instruments of smaller dimensions and, therefore, also of smaller calorific effect. They expect the instruments to be "friendly" to the user: they must not be noisy, the instructions for use must be lucid and should also be available in German, and the instruments must be easily accessible for servicing. It would be useful if the injection systems and the joints for pipes and tubes were standardized. The systems should be constructed in modules and be multi-purpose. There is still a demand for more efficient detectors, particularly with regard to trace determinations.

In LC we expect improvements in efficiency by further optimization of particle size distributions and column packings. Improvements are also necessary in the flow properties of detector cells and interfaces.

Data systems and integrators ought to be standardized, and communication between operators and data systems should be simplified. Software should involve more flexible programs. During evaluation, for instance, it ought to be possible to select the type of integration subsequent to the recording of the chromatogram. Internal test programs and comparison programs are in demand. Here there should be an important use for microprocessors.

SUMMARY

Many tasks facing industrial analytical chemistry can be solved by chromatography. Modern separation techniques, often combined with up-to-date detection methods, provide analytical systems of great versatility. They are applied to problems arising from industrial activities such as research, development, production and marketing and from environmental considerations, and allow the qualitative, quantitative and behavioural investigation of major components and those at trace or even ultra-trace levels in industrial products.

The needs and opportunities associated with modern chromatographic methods and their development in industrial analytical laboratories are discussed.

THE THIRD NATIONAL SYMPOSIUM ON CHROMATOGRAPHY IN CHINA

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(Received September 24th, 1979)

The Third National Symposium on Chromatography, sponsored by the Chinese Academy of Sciences, was held in Dalian, China, on March 22nd–27th, 1979. The First and Second National Symposia, also sponsored by the Chinese Academy of Sciences, were held in Dalian in October, 1961, and in Lanzhou in October, 1965, respectively, and 45 and 111 papers were presented at them.

After a gap of 14 years, the Third Symposium was attended by 265 delegates and 12 lectures were presented at the Plenary Sessions (the titles and authors are listed in Table I). Each lecture surveyed one area that covered the advances in a particular aspect of chromatography. As these papers will be published elsewhere, they are not included here.

TABLE I
LECTURES PRESENTED AT THE PLENARY SESSIONS

<i>Title</i>	<i>Author</i>
The theoretical basis of column chromatography in multi-component separations	Lu Peichang
The perspective of chromatography in the petroleum industry	Lu Wanzhen
The perspective of GC	Yu Weile
Development of HPLC in medicine	Zhang Hui
The application and development of chromatography in environmental analyses	Huang Junxiong
The application of chromatography in biochemistry	Tao Zongjin
The perspective of GPC	Shi Lianghe
Advances in liquid column chromatography	Hu Zhenyuan
A survey of inorganic HPLC	Gan Weitang
An overview of stationary phases in HPLC	Jiang Zhichang
The perspective of chromatographic instruments	Zhou Baohong
Chromatographic separations and attraction between selective molecules	Tang Xinshuo

The remaining 122 papers were presented in six sessions: (a) theory of gas chromatography (GC), (b) theory of high-performance liquid chromatography (HPLC), (c) chromatographic applications in the petroleum and chemical industries, (d) applications in biomedical analysis, (e) applications in trace analysis and (f) chromatographic instruments.

Various topics were also discussed in eight Seminars. These papers and Semi-

nars demonstrated that chromatography has been widely used and has developed rapidly in China since the Second Symposium.

A summary of some of the techniques and applications described at the Symposium is given in this paper.

The packed column is still used in most gas chromatographic applications. The use for GC purposes of the microparticulate silica employed in HPLC has been studied. With carbon dioxide as the carrier gas, a column performance of over 40,000 plates per metre has been achieved and this method has been used for the rapid analysis of gaseous hydrocarbons (Fig. 1). A column packed with a thin coating of carbon molecular sieve on DT-6201 (Kieselguhr support) has been used for this separation, with a shorter time and a lower column temperature (Fig. 2).

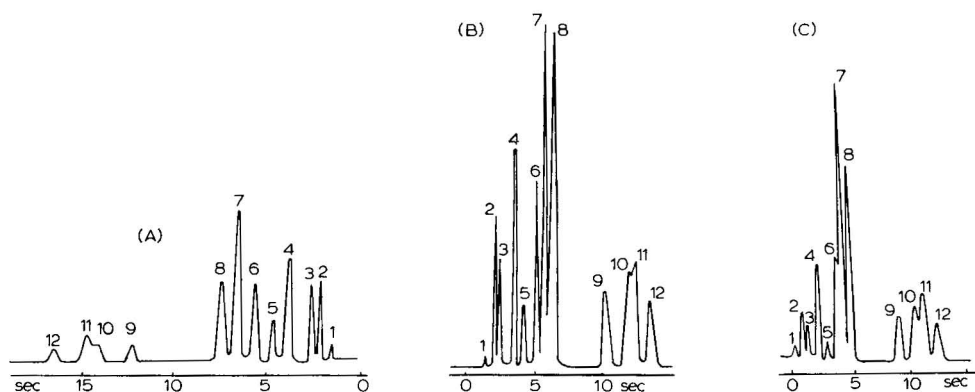


Fig. 1. Chromatogram of C_1 - C_4 hydrocarbons. Column, 10 cm \times 2 mm I.D.; stationary phase, $7 \pm 2 \mu\text{m}$ silica; carrier gas, CO_2 . Column temperature: (A) 19° ; (B) 30° ; (C) 40° . Column inlet pressure: (A) 28.8 atm; (B) 28.5 atm; (C) 30 atm. Peaks: 1 = CH_4 ; 2 = C_2H_6 ; 3 = C_2H_4 ; 4 = C_3H_8 ; 5 = C_2H_2 ; 6 = C_3H_6 ; 7 = iso- C_4H_{10} ; 8 = *n*- C_4H_{10} ; 9 = butene-1; 10 = *trans*-butene-2; 11 = butadiene-1,3; 12 = *cis*-butene-2.

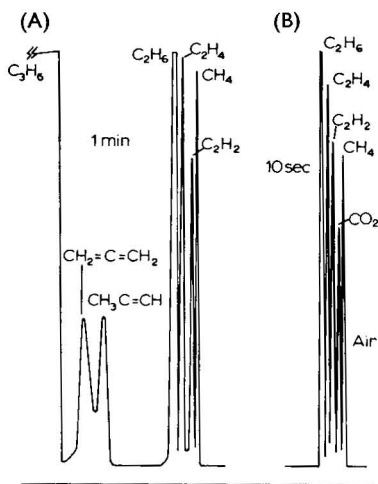


Fig. 2. Chromatogram obtained with a packed column with a thin coating of carbon molecular sieve on DT-6201. Column, 0.5 m \times 4 mm I.D. Column temperature: (A) 150° ; (B) 120° . Carrier gas: (A) N_2 ; (B) H_2 . Detector: (A) FID; (B) TCD.

Various kinds of stationary phase for GC have been developed in China, such as the liquid crystalline phases that have been used recently for the separation of isomers, as shown in Fig. 3. Bentone-34, modified with methylbenzylsilicone (about 24% benzyl), can be used to separate almost all chloro-substituted benzenes with

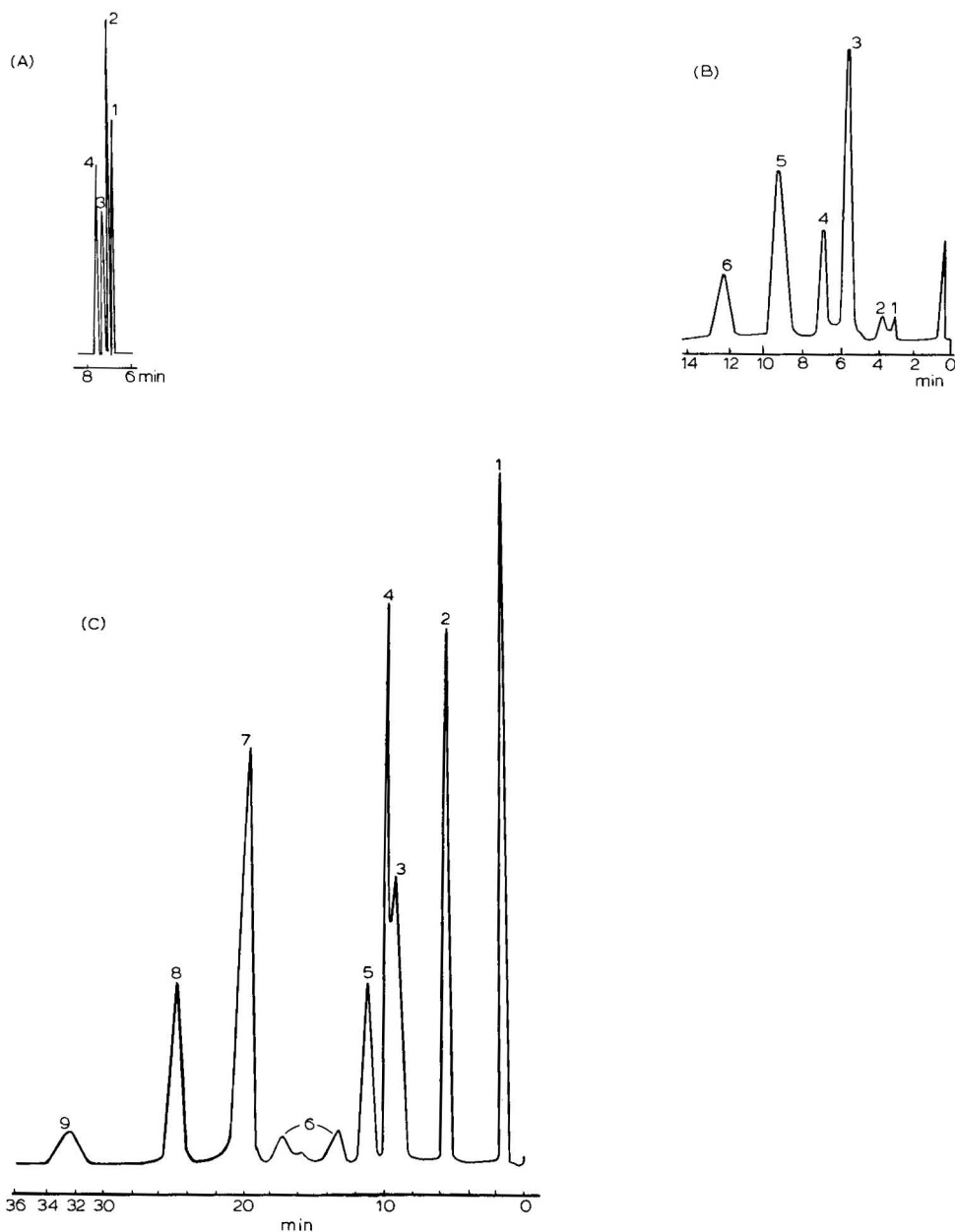


Fig. 3.

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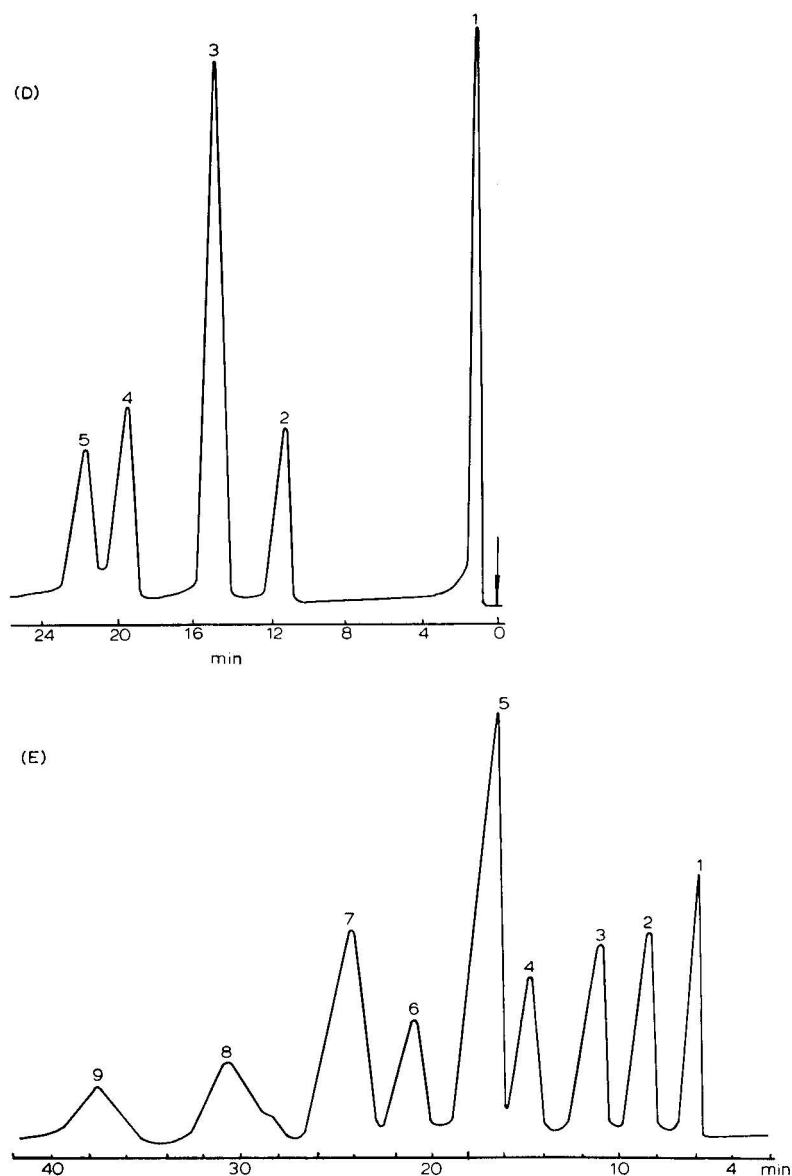


Fig. 3. Results obtained with a Chinese liquid crystal as the stationary phase. (A) Separation of industrial xylenes. Column, $43\text{ m} \times 0.28\text{ mm}$ I.D.; stationary phase, M-201; column temperature, 67° ; carrier gas, N_2 . Peaks: 1 = ethylbenzene; 2 = *m*-xylene; 3 = *p*-xylene; 4 = *o*-xylene. (B) Separation of diethylenebenzenes. Column, $4\text{ m} \times 3\text{ mm}$ I.D.; stationary phase, 7% M-104 on Chromosorb W NAW; column temperature, 109° ; carrier gas, H_2 , 18 ml/min. Peaks: 1 = *m*-diethylenebenzene; 2 = *p*-diethylenebenzene; 3 = *m*-ethylethylenebenzene; 4 = *p*-ethylethylenebenzene; 5 = *m*-diethylenebenzene; 6 = *p*-diethylenebenzene. (C) Separation of polynuclear aromatics. Column, $2\text{ m} \times 3\text{ mm}$ I.D.; stationary phase, 10% PBHxB on DT-101; column temperature, 190° ; carrier gas, H_2 , 20 ml/min. Peaks: 1 = solvent; 2 = naphthalene; 3 = β -methylnaphthalene; 4 = α -methylnaphthalene; 5 = diphenyl; 6 = dimethylnaphthalene; 7 = acenaphthene; 8 = fluorene oxide; 9 = fluorene. (D) Separation of cresols. Column, $3\text{ m} \times 3\text{ mm}$ I.D.; stationary phase, 10% PBOB on DT-101; column temperature, 118° ; carrier gas, H_2 , 20 ml/min. Peaks: 1 = methanol; 2 = *o*-hydroxybiphenyl; 3 = *o*-cresol; 4 = *m*-cresol; 5 = *p*-cresol. (E) Separation of pyridines. Column, $2\text{ m} \times 3\text{ mm}$ I.D.; stationary phase, 10% BPAPA on DT-101; column temperature, 112° ; carrier gas, N_2 , 22 ml/min. Peaks: 1 = pyridine; 2 = α -methylpyridine; 3 = 2,6-dimethylpyridine; 4 = β -

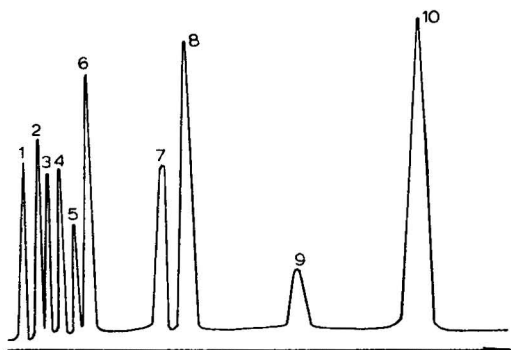


Fig. 4. Separation of chloro-substituted benzenes. Column, glass, 206 cm \times 3 mm I.D.; stationary phase, 6% Bentone-34 + 11% phenyl methyl silicone on DT-101; column temperature, 155°; carrier gas, N₂, 40 ml/min. Peaks: 1 = chlorobenzene; 2 = *p*-dichlorobenzene; 3 = *m*-dichlorobenzene; 4 = 1,3,5-trichlorobenzene; 5 = *o*-dichlorobenzene; 6 = 1,2,4-trichlorobenzene; 7 = 1,2,3,5-tetrachlorobenzene; 8 = 1,2,3-trichlorobenzene; 9 = unknown; 10 = pentachlorobenzene.

boiling points in the range 132–309° (except for 1,2,3,5- and 1,2,4,5-tetrachlorobenzene), as shown in Fig. 4.

The variation of the retention values of compounds on polar and non-polar stationary phases was used for the identification of the peaks of some methyl esters of fatty acids in *Xanthocoras sorbifolia* Bge, as shown in Figs. 5 and 6.

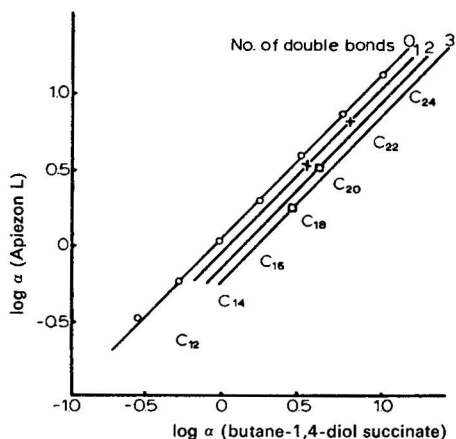


Fig. 5. Plot of logarithm of relative retention ($\log \alpha$) on two columns with 10% butane-1,4-diol succinate on Shimalite and 10% Apiezon L on Shimalite as stationary phase. Column temperatures: butane-1,4-diol, 198°; Apiezon L, 235°.

The flame photometric detector (FPD) has been used for the quantitative analysis of various sulphur compounds. The results showed that different sulphur compounds gave the same response based on sulphur atoms (Fig. 7), and therefore one sulphur compound could be used for the calibration of various other sulphur compounds. By using an electron-capture detector (ECD) in the gas chromatograph made by the Analytical Instruments Factory, Shanghai, with pre-treatment of the sample with a chelating agent, trace metals could be analysed, as shown in Table 2.

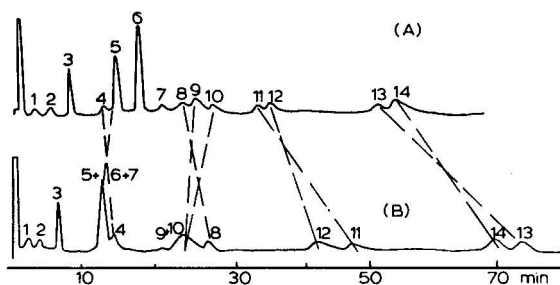


Fig. 6. Separation of fatty acid methyl esters in *Xanthocoras sorbifolia* Bge. Column, 2 m \times 3 mm I.D. Stationary phase: (A) 10% butane-1,4-diol succinate on Shimalite; (B) 10% Apiezon L on Shimalite. Column temperature: (A) 198°; (B) 235°. Peaks: 1 = C₁₂; 2 = C₁₄; 3 = C₁₆; 4 = C₁₈; 5 = C_{1:18}; 6 = C_{2:18}; 7 = C_{3:18}; 8 = C₂₀; 9 = C_{1:20}; 10 = C_{2:20}; 11 = C₂₂; 12 = C_{1:22}; 13 = C₂₄; 14 = C_{1:24}.

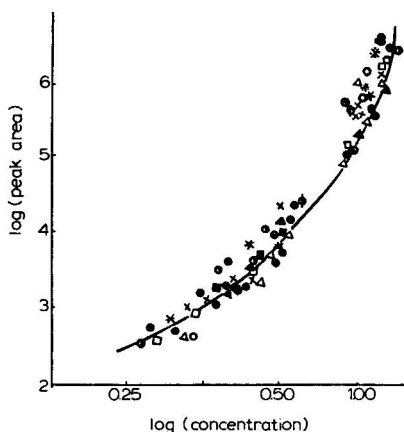


Fig. 7. FPD molar response of various sulphur compounds. Δ , *n*-Propylmercaptan; \circ , 2-methylpropanethiol-1; \blacksquare , *n*-butylmercaptan; \blacktriangle , *n*-amylmercaptan; \square , 2-hexylmercaptan; \circ , thiophene; +, tetrahydrothiophene; \times , 2,2-dimethylpropanethiol-1; \otimes , isobutylmercaptan; \bullet , *n*-hexylmercaptan; $*$, 3-hexylmercaptan; \otimes , dimethyl disulphide; \diamond , diethyl sulphide.

TABLE 2

ANALYSIS OF TRACE METALS

Element	Reagent	Limit of detection (g)
Be	Trifluoroacetylacetone-benzene	$5 \cdot 10^{-12}$
Cr	Trifluoroacetylacetone-benzene	$1 \cdot 10^{-12}$
Se	4-Nitro- <i>o</i> -diphenylamine	$5 \cdot 10^{-11}$

A gas chromatograph combined with a microwave plasma spectrometer has been used to analyse a nine-component mixture of CH₃I, (CH₃)₂CO, CCl₄, C₂H₄Cl, *n*-C₉H₂₀, C₃H₇I, CH₂Br₂, C₂H₅NO₂ and C₂H₄Br₂ (Figs. 8 and 9).

GC has been used to analyse the impurities in chlorobutadiene and ethyl acetate, as shown in Figs. 10 and 11, and steroids in human urine have also been studied by this technique (Fig. 12).

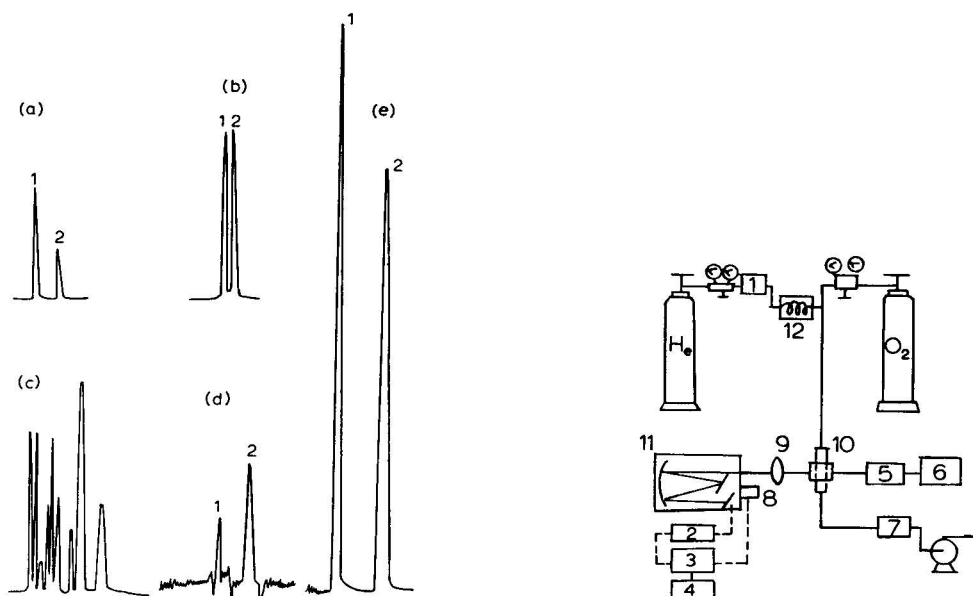


Fig. 8. Chromatogram obtained with microwave plasma detector. (a) Path of I_2 . Peaks: 1 = CH_3I ; 2 = $CH_3CH_2CH_2I$. (b) Path of Cl_2 . Peaks: 1 = CCl_4 ; 2 = $C_2H_4Cl_2$. (c) FID signal. (d) Path of O_2 . Peaks: 1 = CH_3COCH_3 ; 2 = $C_2H_5NO_2$. (e) Path of Br_2 . Peaks: 1 = CH_2Br_2 ; 2 = C_2H_4Br .

Fig. 9. Schematic diagram of microwave plasma detector. 1 = Purifier of rare gas; 2 = vibrator; 3 = lock-in amplifier; 4 = recorder; 5 = reflectometer; 6 = microwave generator; 7 = stabilized gaseous pressure; 8 = photomultiplier tube; 9 = lens; 10 = quartz tube; 11 = monochromator; 12 = column.

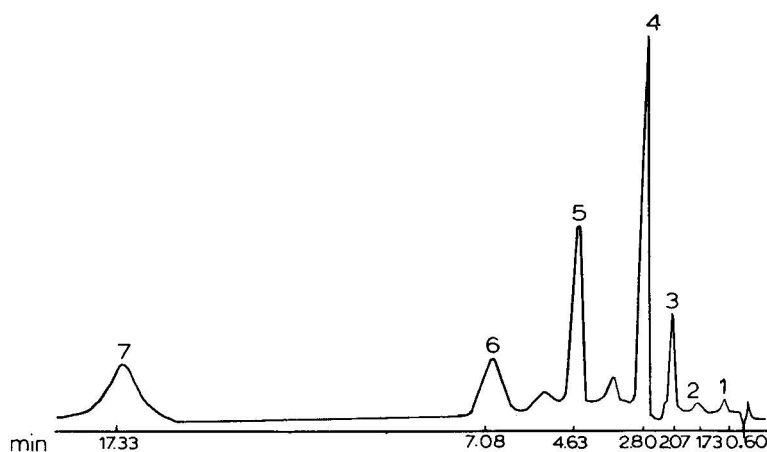


Fig. 10. Separation of trace components in chlorobutadiene. Column, glass, 349×0.6 cm I.D.; stationary phase, propylene glycol-dipropionitrile ether-liquid paraffin (15:5:100); column temperature, 95° ; carrier gas, H_2 , 100 ml/min; detector, TCD. Peaks: 1 = air; 2 = vinylacetylene; 3 = acetaldehyde; 4 = chlorobutadiene; 5 = divinylacetylene; 6 = methyl vinyl ketone; 7 = 1,3-dichlorobutene-2.

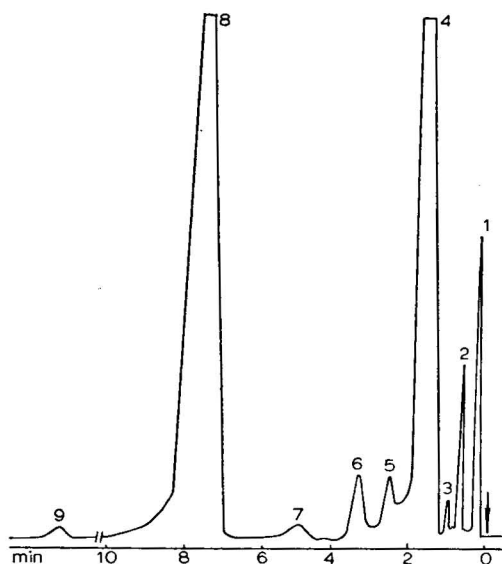


Fig. 11. Separation of minor components in ethyl acetate. Column, 2 m \times 3 mm I.D.; stationary phase, Carbowax 400-*m*-phthalic acid-dinonyl phthalate (5:6:30); column temperature, 102°; carrier gas, N₂, 35 ml/min. Peaks: 1 = acetylene; 2 = acetaldehyde; 3 = acetone; 4 = ethyl acetate; 5 = benzene; 6 = crotonaldehyde; 7 = unknown; 8 = acetic acid; 9 = unknown.

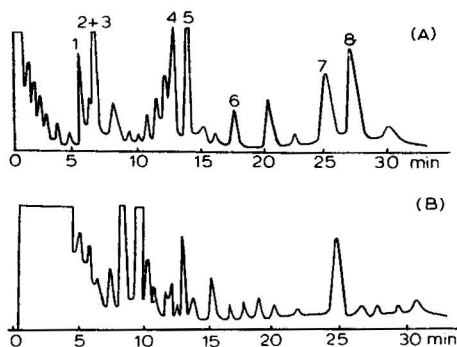


Fig. 12. Analysis of steroids in human urine. Column, glass, 2 m \times 4 mm I.D.; stationary phase, OV-101; column temperature, 190–250°, at a rate of 2°/min; detector, FID. (A) Standard mixture; (B) woman's urine. Peaks: 1 = androsterone; 2 = dehydroepiandrosterone; 3 = 5-isoandrosterone; 4 = pregnanediol; 5 = pregnenolone; 6 = desoxycorticosterone; 7 = corticosterone; 8 = hydrocortisone.

Capillary column GC is being used increasingly to solve many analytical problems, and many papers were presented at this Symposium describing open-tubular columns and chromatographs made in this country.

A capillary column packed with alumina has been used to analyse C₁–C₅ hydrocarbons within 34 min, as shown in Fig. 13.

A stainless-steel capillary tube coated with a thin layer of molecular sieve 13X has been used to separate alkanes and naphthenes (Fig. 14).

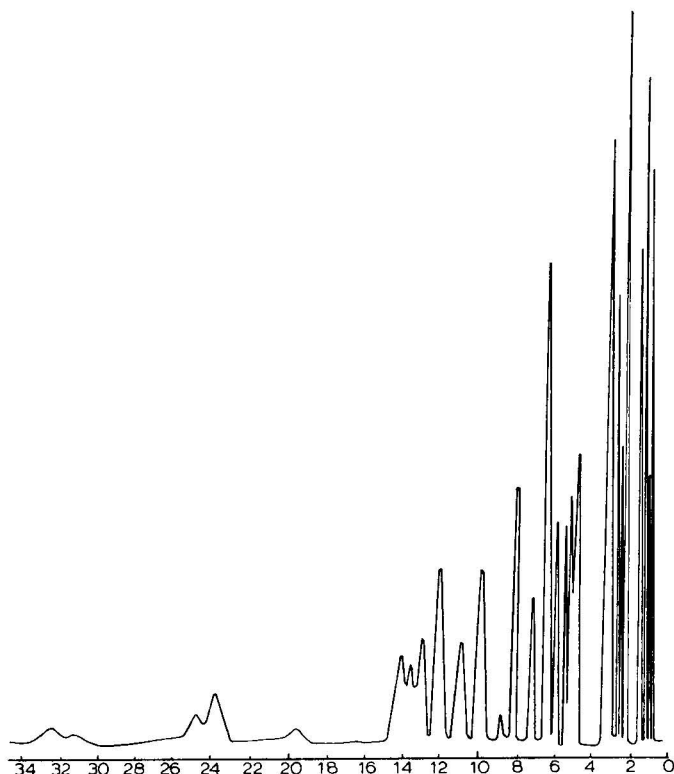


Fig. 13. Chromatogram of C_1 - C_5 hydrocarbons obtained with a packed capillary column. Column, $10.3\text{ m} \times 0.25\text{ mm}$ I.D., capillary column packed with Al_2O_3 ; column temperature, 78° ; carrier gas, N_2 ; detector, FID.

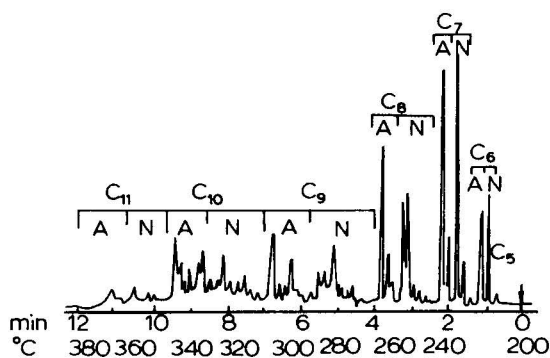


Fig. 14. Separation of C_5 - C_{11} alkanes (A) and naphthenes (N) with a PLOT column. Column, $6\text{ m} \times 0.35\text{ mm}$ I.D., coated with molecular sieve 13X; column temperature, programmed from 200° to 380° at $20^\circ/\text{min}$; carrier gas, H_2 , $5\text{ ml}/\text{min}$.

A glass wall-treated open tubular (WTOT) column obtained by "etching" with hydrogen chloride gave a good performance, as shown in Table 3. By using capillary GC-mass spectrometry (MS), bicyclo[3,3,0]octane and bicyclo[4,3,0]nonane have been identified in Dagang petroleum, as shown in Fig. 15.

TABLE 3

PERFORMANCE OF WTOT COLUMN PREPARED BY "ETCHING" WITH HYDROGEN CHLORIDE

	Stationary phase		
	OV-101	OV-17	Carbowax 20M
Column length (m)	27	25	30
Liquid film thickness (μm)	0.5-0.6	0.2-0.3	0.5-0.6
Sample	$n\text{-C}_{12}\text{H}_{26}$	$n\text{-C}_{12}\text{H}_{26}$	1,3,5-Trimethylbenzene
N_{eff}/m	2000	2000	1500
k'	4.7	3.4	3.7

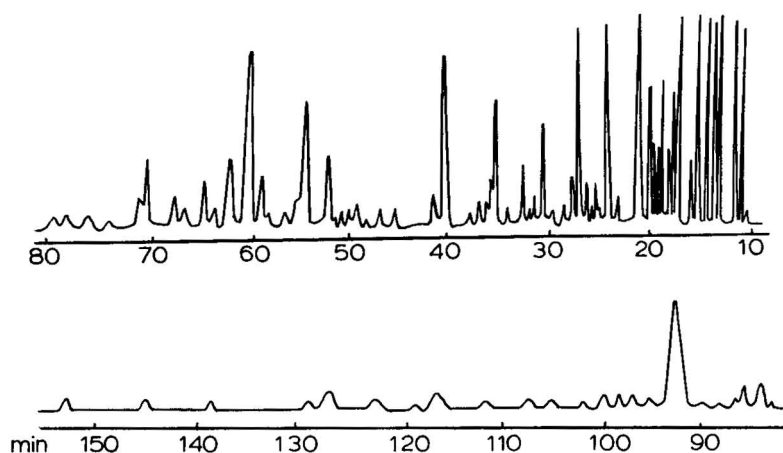


Fig. 15. Chromatogram of straight gasoline (60-165°) from Dagang.

TABLE 4

PERFORMANCE OF SCOT COLUMNS MADE IN CHINA

Parameter	Stationary phase		
	Squalane	SE-30	Carbowax 20M
Column dimensions	51 m \times 0.27 mm I.D.	31 m \times 0.24 mm I.D.	33.5 m \times 0.30 mm I.D.
Support	DT-405	DT-405	DT-405
Column temperature ($^{\circ}\text{C}$)	50	220	140
Carrier gas	N_2	N_2	H_2
Flow-rate (cm/sec)	10.4	10	9.6
Sample	$n\text{-C}_7$	$n\text{-C}_{19}$	$n\text{-C}_9\text{OH}$
N/m	3100	4500	2800
N_{eff}/m	1600	3100	2200
k'	2.7	4.8	6.7
Utilization of theoretical column efficiency (UTE) (%)	65	91	73

Some support-coated open tubular (SCOT) columns have been produced in China, and their properties are given in Table 4. A SCOT column has been used in the analysis of lavender oil (Fig. 16).

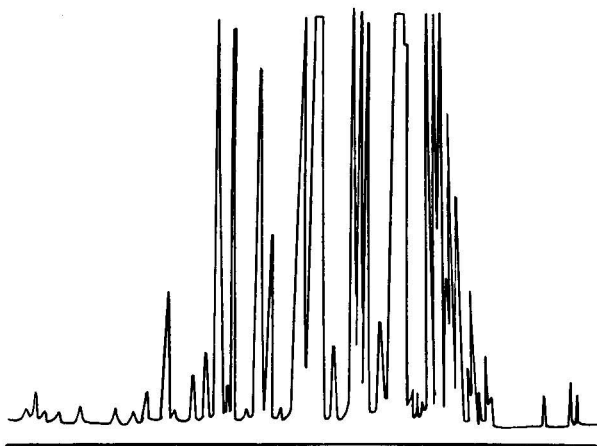


Fig. 16. Chromatogram of lavender oil obtained with a SCOT column. Column, 22 m \times 0.3 mm I.D.; stationary phase, SE-30; column temperature, programmed from 72° to 165° at 4°/min; carrier gas, N₂; detector, FID.

A SCOT column coated with squalane has been used to analyse a naphtha fraction of b.p. 36–125°. Seventy-four components were identified by using the Kováts' retention index method and 69 components were determined quantitatively by using the retention time–peak height method. A SCOT column with more than 400,000 theoretical plates was used in a modified 2305 gas chromatograph to separate a cracking gasoline fraction into 239 peaks (Fig. 17); 256 components were identified by using a combination method of Kováts' retention index method, authentic substances, MS and catalytic hydrogenation.

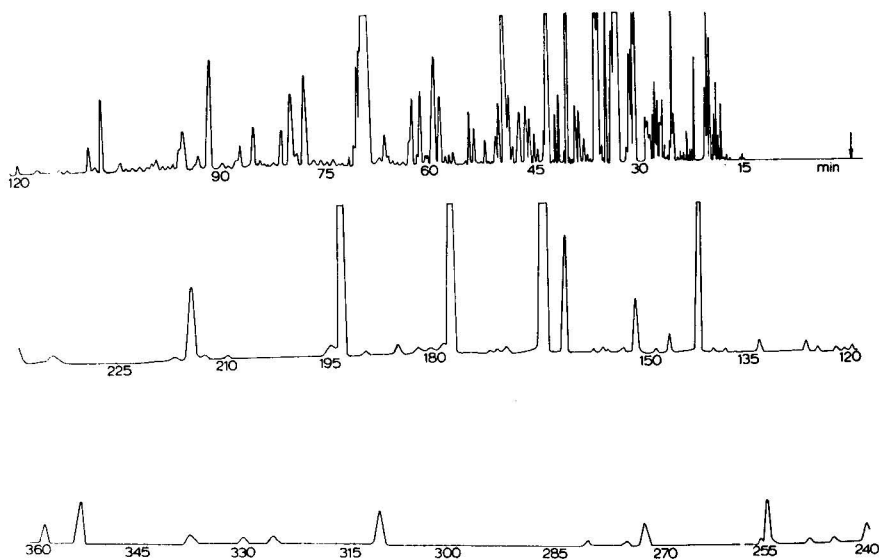


Fig. 17. Chromatogram of cracking gasoline (60–150°) obtained with a SCOT column.

Also on a SCOT column, a straight line for the relationship between the peak width ($2\Delta t_{\frac{1}{2}}$) and retention time (t_R) that passed through the origin was obtained (Fig. 18).

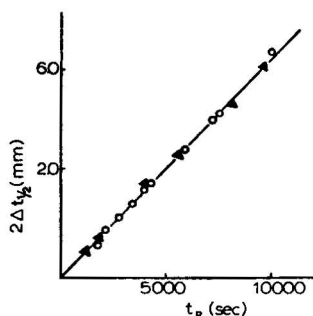


Fig. 18. Relationship between peak width ($\Delta t_{1/2}$) and retention time (t_R) on a SCOT column.

High-performance liquid chromatographs and stationary phases such as microparticulate silica and chemically bonded silica packings are being produced in China. A linear relationship was observed between peak width and retention volume (V_R) not only for different solutes with different capacity ratios (k') on a given column, but also on different columns with different inner diameters according to the equation

$$2\Delta V_{\frac{1}{2}} = a' + bV_R \quad (1)$$

The column of detector system had a great influence on a' . As shown in Table 5, a highly efficient column was obtained with a column diameter of only 2 mm by using a detector system with a small volume.

TABLE 5
VALUES OF CONSTANTS IN EQN. 1 USING DIFFERENT DETECTOR SYSTEMS

No. of detector system	Dead volume of detector system (μl)	a' (μl)	b	Correlation coefficient, r
1	21	20.5 ± 1.5	0.024	0.996
2	11	11 ± 1.3	0.023	0.996
3	2	1.7 ± 1.2	0.022	0.995

By using microparticulate silica and improving the packing method for the Familie 100 liquid chromatograph, a column efficiency of more than $10 \cdot 10^3$ plates for an 11 cm \times 0.5 mm I.D. column was obtained. The graph of peak width *versus* retention volume was a straight line passing through the origin.

A chemically bonded packing based on aggregate silica beads has been evaluated with an ALC/GPC 244 liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.), as shown in Table 6.

TABLE 6

COLUMN PERFORMANCES WITH CHEMICALLY BONDED PACKINGS BASED ON AGGREGATE SILICA BEADS (ASB)

Packing	Column dimensions (mm)	Solute	Eluent	Flow-rate (ml/min)	N per 10 cm
ASB	100 × 3.2	Methoxybenzene	<i>n</i> -Hexane	0.35	$3.7 \cdot 10^3$
ASB-CH	100 × 5	Diphenyl	Methanol-water (80:20)	0.6	$1.5 \cdot 10^3$
ASB-CN	100 × 5	Diphenylamine	<i>n</i> -Hexane-isopropanol (97:3)	1.0	$2.6 \cdot 10^3$
ASB-R ₂ O	100 × 5	Butanone hydrazone	<i>n</i> -Hexane-isopropanol (97:3)	1.2	$1.3 \cdot 10^3$

Carbowax 400 chemically bonded silica beads have been compared with Durapak CW-400, the capacity ratios of six compounds being determined on each column. A linear relationship was found, as shown in Fig. 19.

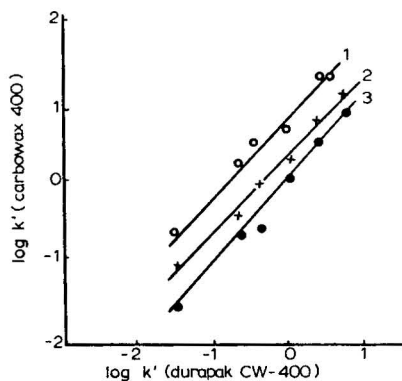


Fig. 19. Relationship between retention values of different solutes on two columns. 1 = Silica beads (37–55 μ m); 2 = silica beads-PEG 400 (Shanghai); 3 = silica beads-PEG 400 (Tianjing).

Chemically bonded silica with a particle size of 30 μ m was used to determine carbonyl-group containing compounds in water (Fig. 20). The time of analysis was long. The peaks were identified from the retention times and IR and UV spectra.

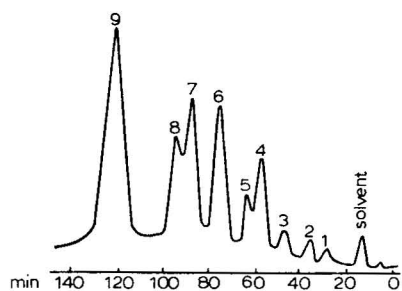


Fig. 20. Chromatogram of phenylhydrazones of the following compounds. 1 = Unknown; 2 = 2-butanone; 3 = acetone; 4 = methacrolein; 5 = crotonaldehyde; 6 = acraldehyde; 7 = acetaldehyde; 8 = unknown; 9 = formaldehyde.

Heavy metals and rare earth elements have been separated with cation-exchange resins using a coulombic detector (Fig. 21).

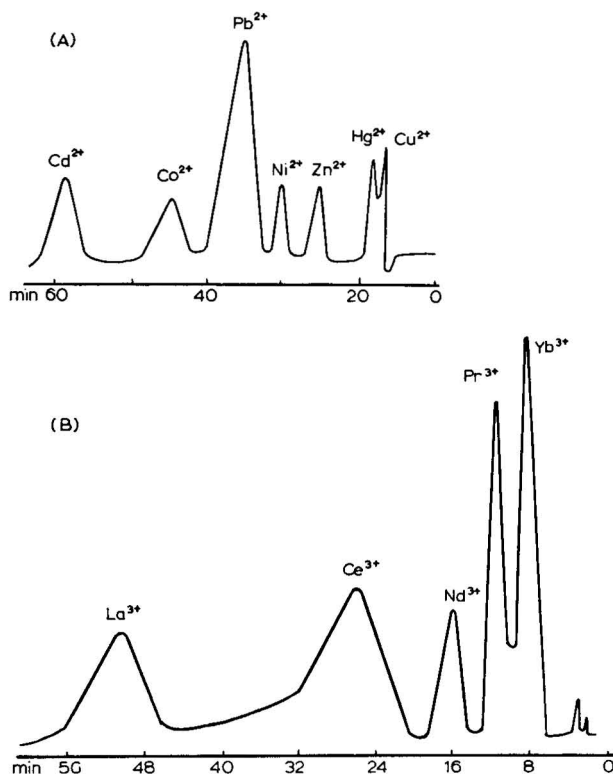


Fig. 21. Chromatograms of (A) heavy metals and (B) rare earth elements.

Microparticulate stationary phases are being used increasingly. For example, YWG (5 μ m) was used to separate maytansine and maytanprine from *Maytenus* from Yunnan province (Fig. 22).

Chromatography is being widely used for the analysis of Chinese drugs, such as *Baeckea frutescens* Linn., *Artemisia cina* Beng., *Angelica sinensis* (Oliv.) Diels, *Levisticum officinale* Koch, *Rhododendron*, *Schisandra sphenanthera* Rehd. et Wils. and *Salvia miltiorrhiza* Bung.

Various HPLC column systems have been utilized for the qualitative analysis of pesticides. By using a microparticulate packing, six organophosphorus pesticides could be separated under various conditions within 5–20 min. The separation conditions and relative retention values are given in Table 7 and Fig. 23.

The dependence of k' on carbon number was established by using YWG-CH as the stationary phase and methanol as eluent, as shown in Fig. 24.

Liquid chromatograph SY-01 with a UV detector (Analytical Instrument Factory, Beijing) and with the chemically bonded stationary phase YWG-CN (10 μ m)

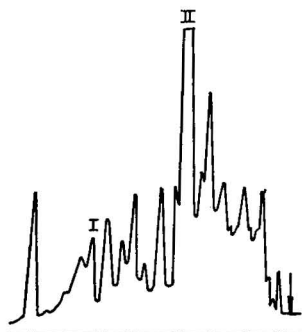


Fig. 22. Separation of maytansine (I) and maytanprine (II) by HPLC. Column, 15 m \times 1 cm I.D.; stationary phase, YWG (5 μ m); eluent, dichloromethane-methanol (98:2); flow-rate, 3 ml/min. Structures:

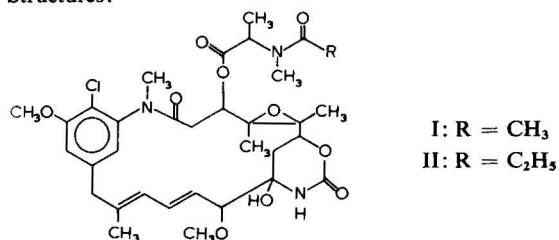


TABLE 7

RELATIVE RETENTION VALUES OF PESTICIDES

Temperature: 230°.

Pesticide	Conditions		
	I*	II**	III***
Parathion	1.00	1.00	1.00
Imidan	1.39	2.48	3.33
Gardona	1.81	2.18	4.95
Pyridaphenthion	2.17	6.76	10.83
Ethyl guthion	2.49	1.88	3.33
Phosalone	2.87	1.36	0.72

* Chromatogram A (Fig. 23); stationary phase, YWG-C₆H₅; eluent, methanol-water (50:50).

** Chromatogram B (Fig. 23); stationary phase, YWG-CN; eluent, isopropanol-*n*-heptane (3:97).

*** Chromatogram C (Fig. 23); stationary phase, YWG (5 μ m); eluent, isopropanol-*n*-heptane (5:95).

(Second Chemical Reagent Plant, Tianjing) has been used to analyse polynuclear aromatics in air, as shown in Fig. 25. The flow-rate of air was 12–14 m³/h and the time for sampling was 6–18 h.

Fatty acids have been analysed on YWG-CN (10 μ m) with a refractive index detection using a Hitachi 635 high-performance liquid chromatograph (Fig. 26).

Nucleic bases have also been analysed by HPLC (Fig. 27).

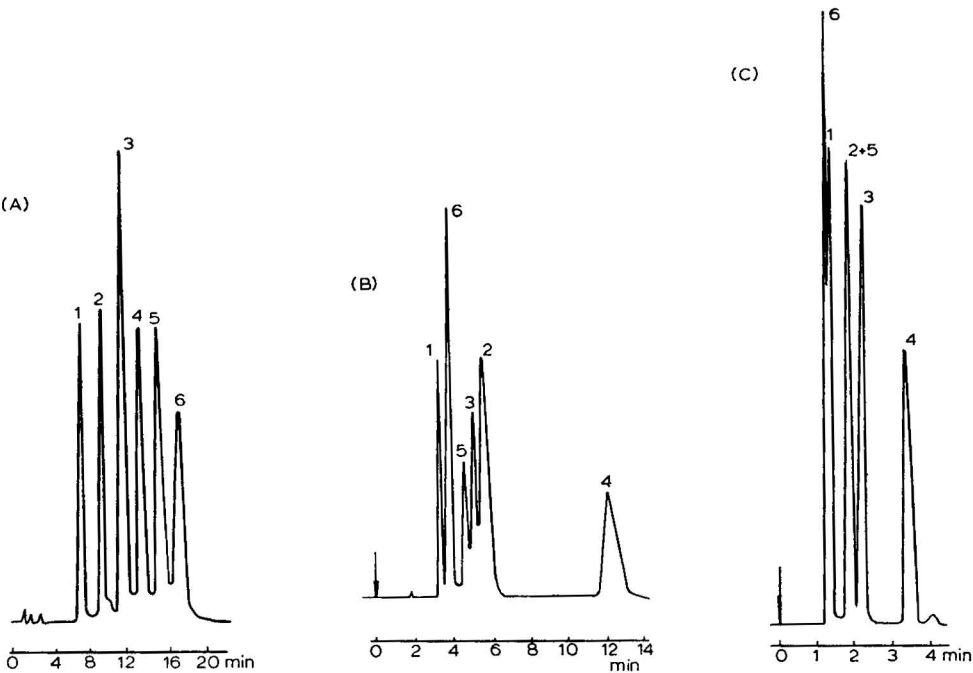


Fig. 23. Chromatograms of pesticides on micro-particulate packings: (A) YWG-C₆H₅ (5 μm); (B) YWG-CN (5 μm); (C) YWG (5 μm). For peaks, see Table 7.

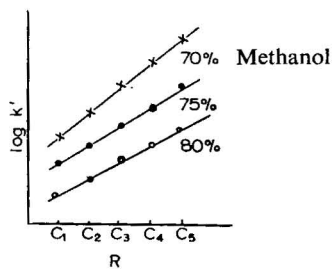
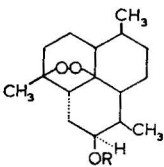


Fig. 24. Relationship between $\log k'$ and carbon number of the alkyl group (R) of the following compound:



Stationary phase: YWG-CN.

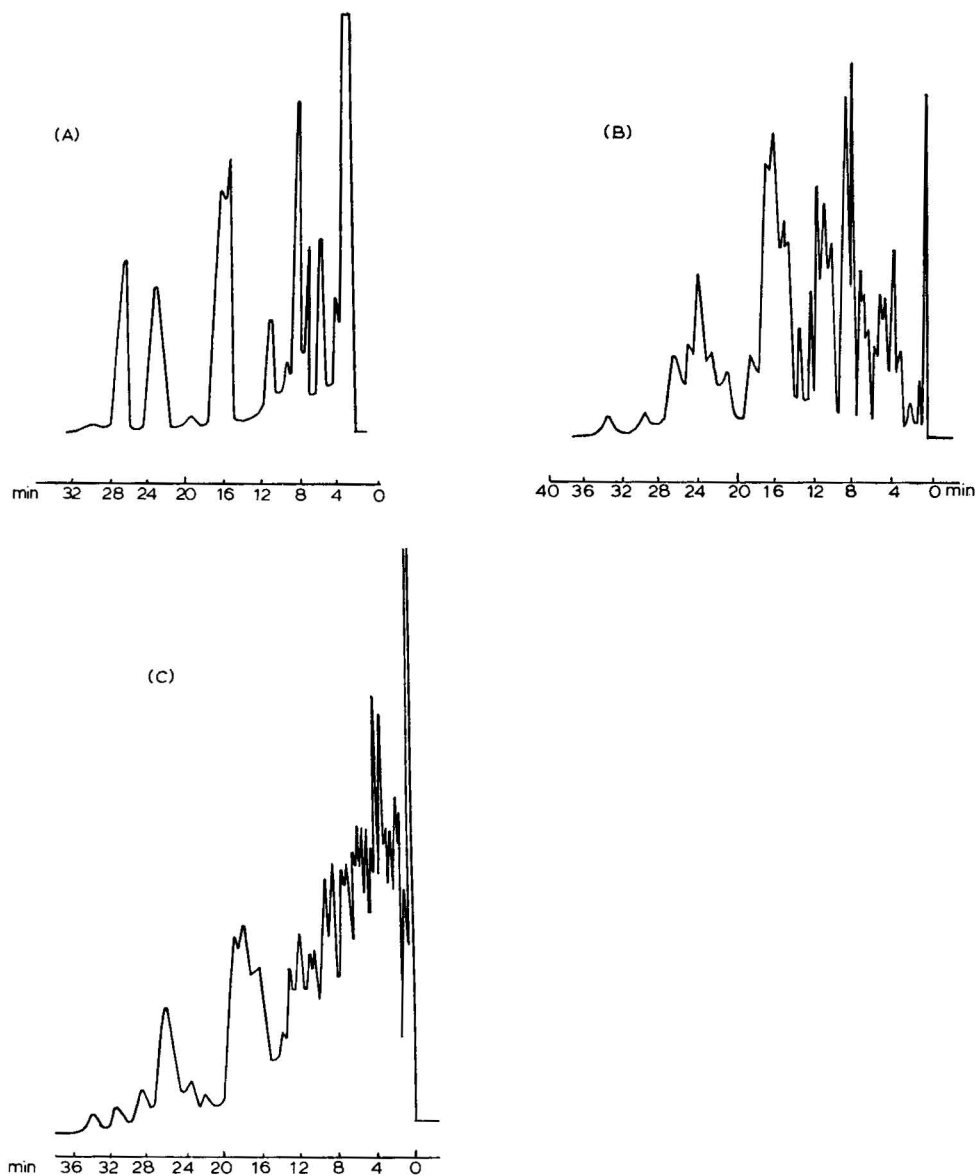


Fig. 25. Separation of polynuclear aromatic hydrocarbons in air by HPLC. Column, 20 cm \times 5 mm I.D.; stationary phase, YWG-CN (10 μ m); eluent, methanol-water (75:25); flow-rate, 1.1 ml/min; column temperature, 40°; detection, UV (254 nm). (A), Standard mixture; (B), sample 1 (near factory); (C), sample 2 (far from factory).

YWG (5 μ m) has been used to separate 17 PTH amino acids (Fig. 28) and YWG-CN has been also used to separate 18 PTH amino acids (Fig. 29). The responses from two detectors were used for quantitative analyses of components that were not separated on the column.

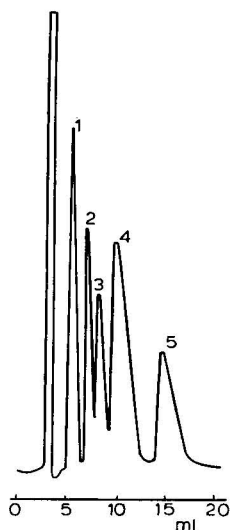


Fig. 26. Separation of C_{12} - C_{18} fatty acids by HPLC. Column, 250×4 mm I.D.; stationary phase, YWG-CN ($10 \mu\text{m}$); eluent, acetonitrile-methanol-water (15:70:15); flow-rate, 1 ml/min; detection, refractive index. Peaks: 1 = lauric acid; 2 = myristic acid; 3 = oleic acid; 4 = palmitic acid; 5 = stearic acid.

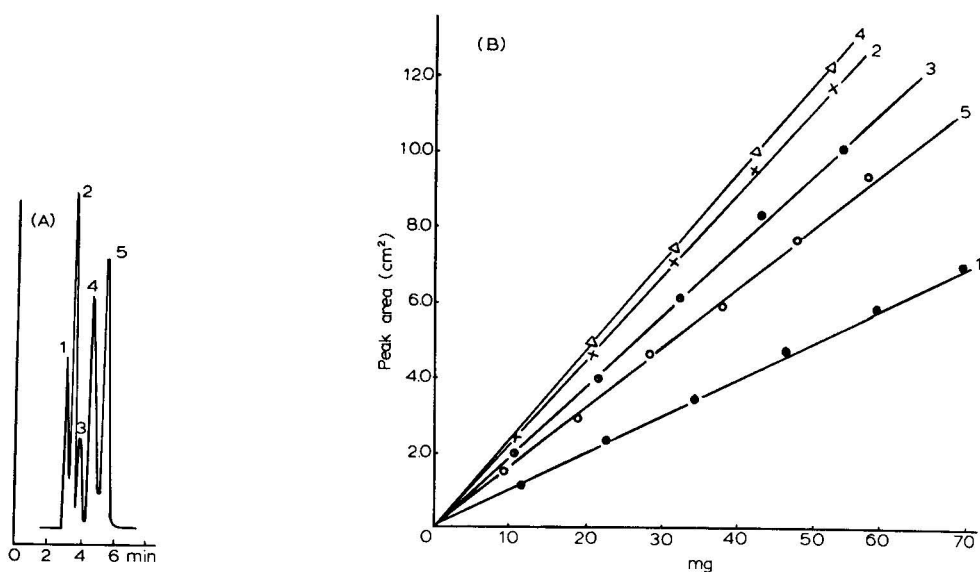


Fig. 27. Separation of nucleic bases by HPLC. Column, 250×3.0 mm I.D.; stationary phase, YWG-CH ($10 \mu\text{m}$); eluent, methanol- $0.01 M \text{KH}_2\text{PO}_4$ solution (pH 2.5) (1:20); flow-rate, 0.58 ml/min; inlet pressure, 40 kg/cm^2 . Peaks: 1 = cytosine; 2 = uracil; 3 = guanine; 4 = adenine; 5 = thymine. (A), Chromatogram of sample; (B), calibration graphs.

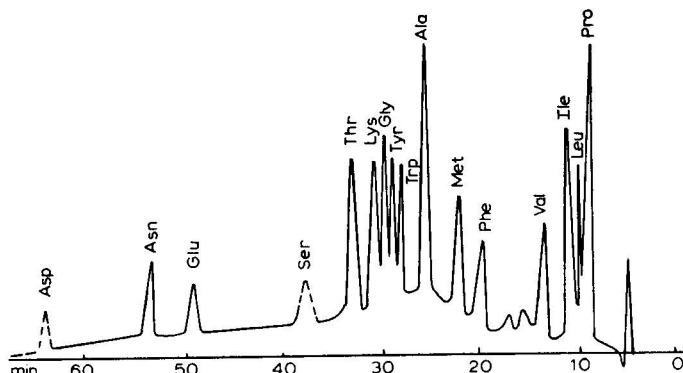


Fig. 28. Separation of PTH-amino acids by HPLC. Column, 220×6 mm I.D.; stationary phase, YWG ($5 \mu\text{m}$); original eluent, 1% isopropanol in *n*-hexane-chloroform (3:1); eluent gradient, 2, 10, 16, 18, 22 and 30% isopropanol in *n*-hexane-chloroform.

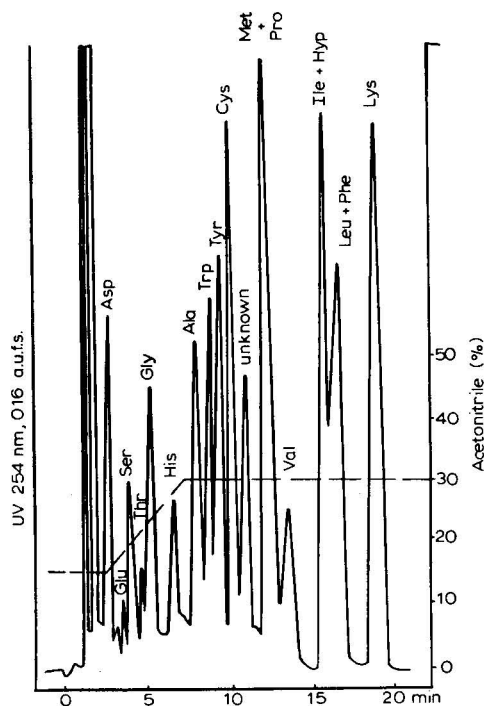


Fig. 29. Separation of PTH-amino acids by HPLC. Column, 50×0.21 cm I.D.; stationary phase, YWG-CH; eluents, (A) 0.001 *M* sodium acetate solution (pH 5.78) and (B) acetonitrile; detection, UV (254 nm).

SUMMARY

A brief survey of some of the advanced techniques for solving analytical problems, which were reported at The Third National Symposium on Chromatography held on March 22–27, 1979 in Dalian, People's Republic of China, is given. A total of 265 delegates attended this meeting and 12 plenary lectures and 122 papers were presented.

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- 4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences*, Ghent, June 16–18, 1976, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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ELECTROPHORESIS

A SURVEY OF TECHNIQUES AND APPLICATIONS

Part A: Techniques

Z. DEYL, Czechoslovak Academy of Sciences, Prague (editor)
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This first volume in a two part set, deals with the principles, theory and instrumentation of modern electromigration methods. The second volume will be concerned with details of applications of electromigration methods to diverse categories of compounds, although a few applications are already discussed in Part A.

Some electromigration methods have become standard procedures because of their extensive use in analytical and preparative separations. These are discussed together with newer developments in the field. Hints are included to help the reader to overcome difficulties frequently arising from the lack of suitable equipment. Adequate theoretical background of the individual techniques is included. A theoretical approach to the deteriorative processes is presented in order to facilitate further development of a particular technique and its application to a special problem.

In each chapter practical realizations of different techniques are discussed and examples are presented to demonstrate the limits of each method. The mathematical and physicochemical background is arranged so as to make it as coherent as possible for both non-professionals such as post-graduate students, and experts using electromigration techniques.

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