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CHROMATOGRAPHIC REVIEWS (Vol. 19, No. 3)

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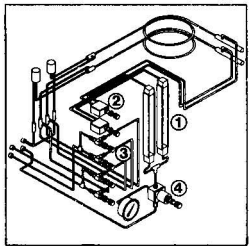

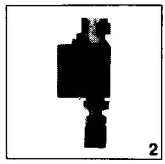

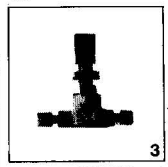

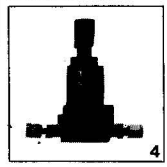

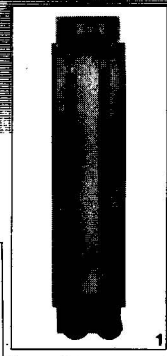

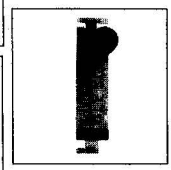

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Vibrational Spectra and Structure

A SERIES OF ADVANCES, VOL. 4

edited by **JAMES R. DURIG,** Department of Chemistry, University of South Carolina, Columbia, South Carolina

1975. 316 pages. US $31.25/Dfl. 75.00. ISBN 0-444-41380-4

One of the greatest needs of science today is for competent people to critically review the recent literature in conveniently small areas and to evaluate the real progress that has been made, as well as to suggest fruitful avenues for future work. The current volume, number 4 in a series now published by Elsevier Scientific Publishing Company, attempts to fulfill these goals. Chapter 1 reports on the complementary infrared and Raman matrix isolation studies of similar chemical systems, and the usefulness of the newer laser-Raman technique is demonstrated relative to the well-established infrared matrix methods. Recent studies on the vibrational spectra and structure of plastic crystals are reviewed in Chapter 2, while Chapter 3 describes methods and applications of intramolecular force field calculations. Chapter 4 continues the theme of Chapter 1 with an example of the valuable use of infrared and Raman matrix isolation techniques. This section reviews the characterization of the products of metal atom-molecule cocondensation reactions studied by these methods. The techniques described should provide the inorganic and organometallic chemist with new chemical pathways for the synthesis and stabilization of chemical species which would have been difficult if not impossible to prepare and study by conventional chemical procedures. Physical chemists, spectroscopists, physicists, and other research scientists who use vibrational spectroscopy in their work should find this volume of immense value.

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11th INTERNATIONAL CHROMATOGRAPHY SYMPOSIUM 1976

The 11th International Symposium on Chromatography is to be held at the new National Exhibition Centre in Birmingham, Great Britain from July 5th-9th 1976.

This is probably the foremost Chromatography meeting in the world and its allied exhibition has achieved considerable fame in its own right.

The Symposium is to be held under the auspices of the Chromatography Discussion Group, the Chromatography and Electrophoresis Group of the Chemical Society (Analytical Division), the Groupement pour l'Avancement des Methodes Spectroscopiques et Physicochimiques d'Analyse (G.A.M.S.), the Arbeitskreis Chromatographie of the Gesellschaft Deutscher Chemiker and the Groupo de Cromatografia of the Real Sociedad Espanola de Fisica y Quimica.

Those who would like to receive information concerning the meeting should contact:

The Executive Secretary
Chromatography Discussion Group
Trent Polytechnic
Burton Street
Nottingham NG1 4BU
Great Britain

Potential exhibitors should contact the Chairman of the Exhibition Committee, at the above address, as soon as possible.

Journal of Chromatography, 113 (1975) 267–282
Chromatographic Reviews

CHREV. 88

PROGRAMMED MULTIPLE DEVELOPMENT

BRIEF REVIEW AND STUDY OF EXTENDED PROGRAMS

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(First received December 31st, 1974; revised manuscript received July 24th, 1975)

CONTENTS

1. SPOT SHAPE AND LOCATION *VERSUS* MULTIPLE DEVELOPMENT TECHNIQUE

Jeanes *et al.*[1] introduced unidimensional multiple chromatography (UMC). UMC improves the resolution obtainable from a given chromatographic system.

In UMC, the solvent front is allowed to advance a given distance, usually the length of the bed of stationary phase. The solvent is then evaporated from the bed, whereupon the same solvent is again allowed to advance the same distance. The process is repeated as often as desired.

With the advent of thin-layer chromatography (TLC)[2], Thoma[3] suggested that the "superb resolving power of UMC" be combined with the convenience of TLC: "A combination of TLC and UMC should prove to be an ideal way to resolve mixtures."

In UMC, the distance of solvent advance is held constant. Alternatively, the solvent can be allowed to advance successively greater distances. Saini[4], for example, in one instance allowed three successive solvent advances through one-third, two-thirds, and finally the whole of the bed length. This clearly improved the separation of the near-front components over that afforded by UMC.

Whether the solvent advance distance is held constant or increased, each solvent advance causes a clearly describable interaction of the solvent front with solute molecules already deposited on the bed. As a solvent front advances through already deposited solute molecules, the solute molecules behind the front advance in the direction of solvent flow at a fraction R_F of the solvent velocity toward those motionless solute molecules still beyond the front. We shall refer to this interaction as spot reconcentration by the solvent advance mechanism.

If the centers of two statistical aggregates of given-R_F molecules initially a

distance X_i apart (as projected along the direction of solvent flow) are traversed by n advancing solvent fronts, these centers become finally only a projected distance X_f apart. The relationship has been described by Jupille and Perry[5,6]:

$$X_f = (1 - R_F)^n X_i \tag{1}$$

Thoma[3] showed that the conventional, single-development R_F is related to the apparent $R_{F,n}$ that exists after the traversals of n UMC advancing solvent fronts, as follows:

$$(1 - R_F)^n = 1 - R_{F,n} \tag{2}$$

It follows that in chromatograms multiply developed by solvent advances alone[7]

$$X_f/X_i = 1 - R_{F,n} \tag{3}$$

X_f/X_i expresses the degree of mutual closing in the direction of solvent flow of the aggregate centers, because $(X_f/X_i)(X_i) = X_f$.

Eqns. 1 and 3 describe spot reconcentration by the solvent advance mechanism. The effects of the operation of this mechanism depend on R_F and, for a given spot, exponentially on the number of solvent advances. The effects, however, do not depend at all on the duration, linear extent, or constancy of the successive solvent advances, as long as all the molecules to be affected are traversed.

During a given solvent advance, the relative positions of identical R_F molecules do not change further once all such molecules have been traversed by the advancing front. Thus eqn. 3 describes spot shape distribution not only in any UMC chromatogram but also in any multiple-development chromatogram involving only solvent advances. No matter how many such multiple developments are carried out, the spot shape distribution is the same. Near the solvent front, where the apparent $R_{F,n}$ is near unity, the spots become almost lines drawn at right angles to the direction of solvent flow. Near the spot origin, however, spots remain almost unchanged.

In contrast, uniform spots result from programmed multiple development (PMD)[5–16]. To illustrate this contrast, two chromatograms differing only in the method of multiple development were prepared: one was developed by UMC, the other by PMD[8]. To show more clearly the effect of these methods of development on spot shape and spot shape distribution, the spots were deposited as rings.

Such comparable UMC and PMD chromatograms are shown in Figs. 1a and b, respectively. The UMC chromatogram shows the dependence of spot shape on apparent $R_{F,n}$ that is predicted by eqn. 3. The PMD chromatogram, on the other hand, shows essentially uniform spots that do not reflect either the spot R_F or the origin shape (or, thus, the origin location).

PMD differs from UMC because, in the former, the spot reconcentrating solvent advance mechanism is supplemented by a second spot reconcentrating mechanism, that of solvent removal. This mechanism is unique to PMD.

In PMD, the chromatographic bed —here, the adsorbent layer on the thin-layer plate— remains at all times in contact with the solvent reservoir. By capillary

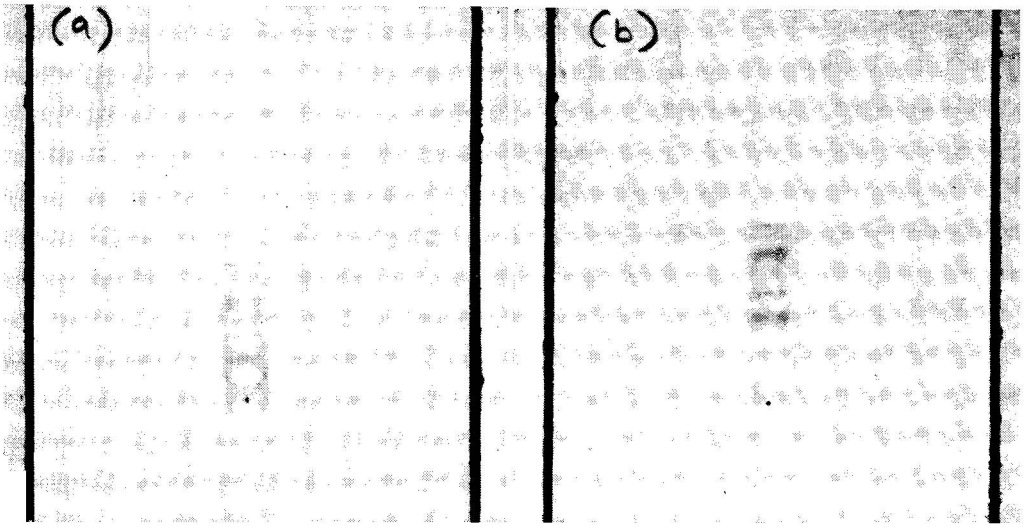

Fig. 1. The spot shapes obtained from solvent advances alone range from "lines" near the solvent front to "circles" near the origin, as shown by the UMC chromatogram (a). Spots reconcentrated by both solvent advances and removals tend to be all "lines", as shown by the PMD chromatogram (b). See also ref. 8. UMC: Three 200-sec advances of benzene through dye mixture. PMD: Three cycles, Mode 1, 100 sec unit time for advance, 100 sec fixed time for removal at power 10. Benzene, same dye mixture.

action the solvent flows at all times toward the solvent front. However, in PMD the rate of evaporation from the bed is controlled. The controlled rates of solvent evaporation during PMD allow the front to move forward into the bed during solvent advance and cause the front to move back toward the solvent reservoir during solvent removals.

In spot reconcentration by the solvent removal mechanism, the solvent flows into, and evaporates from, a receding solvent front. Because of this, solute molecules move into, and are deposited in, the receding front. Solute molecules behind the receding front continue to move toward those motionless solute molecules already deposited from it.

The higher the ratio of the spot velocity to the front recession velocity, the greater the efficiency of spot reconcentration by the solvent removal mechanism[6,8,9].

The rate of solvent removal in PMD can be controlled by any of several means, most commonly radiant heating, a counter-current stream of gas, or both. Usually, when gas is used, the flow-rate is held to a minimum and the gas flow is supplemented by gentle IR radiant heating (see Figs. 2a and b). However, gas alone can be used if the solute is particularly thermally labile.

The suggested actions of the solvent advance and removal mechanisms in reconcentrating spots are shown in Fig. 3.

The mechanisms that reconcentrate spots also align them, as illustrated in Figs. 4a and b.

Thus, the uniformly dense and precisely located spots of the PMD chromatogram do not reflect such normally unfavorable origin characteristics as being spread out or displaced.

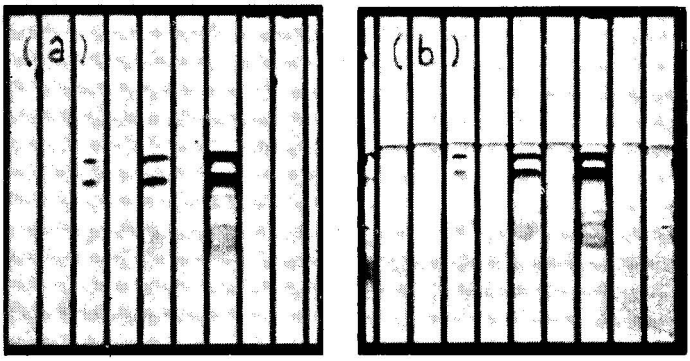

Fig. 2. In PMD, the solvent can be removed by heat alone (a), mainly by gas flush (b), or solely by gas flush. IR radiation (at 5.1 μm wavelength) carries only about 0.05 as much energy per photon as UV at 254-nm wavelength, and so causes little photo-decomposition, but can cause thermal decomposition, in which case solvent is removed mainly or solely by gas. The thermally caused ghost spots shown in (a) are absent in (b)[17]. See also ref. 19. Samples: estrone, estradiol, and estriol, 0.5 mg/ml each, in methanol. Spotted volumes: 1, 5 and 10 μl. Plates: Quantum Q5DF.

To date, most applications of PMD have corresponded to the current applications of TLC; namely, the separation of relatively few components from each other and, at times, from a more complex matrix. PMD has usually been applied to improve a recalcitrant separation or to increase the molecular density within a spot and, thus, the detectability of the spot.

We cite a few examples. An example in which special sensitivity is desired is the moderately difficult separation of deoxycorticosterone (DOC), testosterone, 17-hydroxyprogesterone (17-OH Pr), and progesterone. The difficulty arises because high laboratory humidity plus the use of (possibly) wet nitrogen for solvent removal can deactivate the adsorbent enough to prevent the separation of DOC from 17-OH Pr. In the separation shown in Fig. 5, the solvent was removed with a combination of dried nitrogen and gentle heating.

This separation also serves to illustrate the high reproducibility that is characteristic of PMD spot positions. In PMD, spot positions are measured not from the

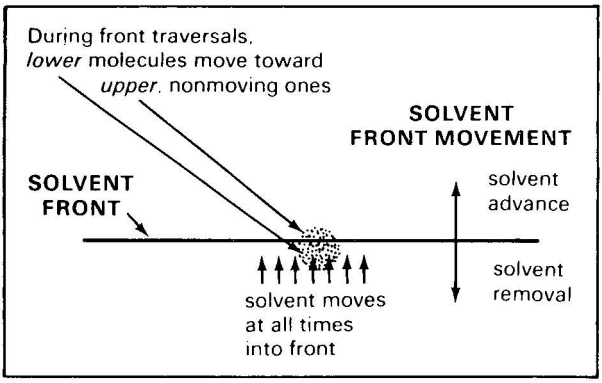

Fig. 3. Spot reconcentration mechanisms in PMD.

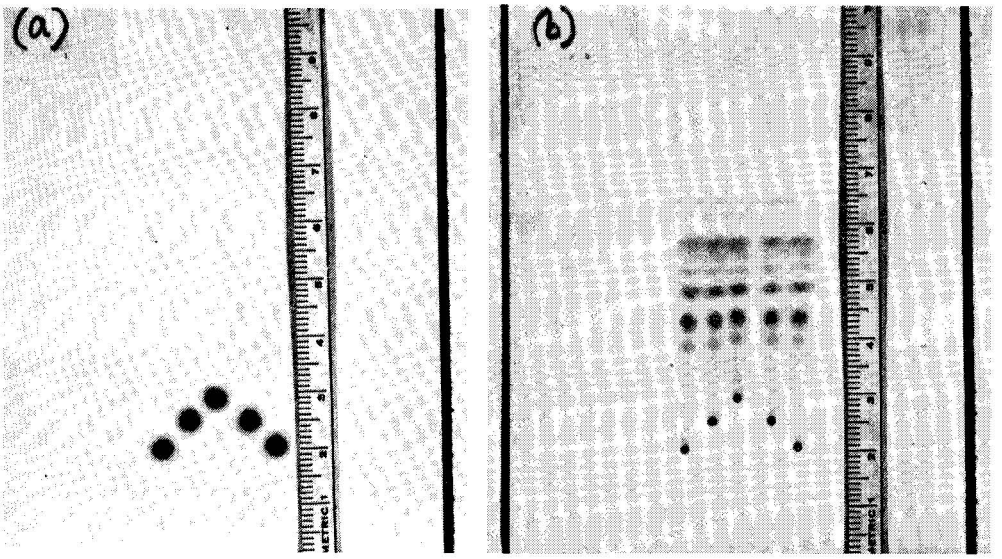

Fig. 4. The PMD mechanisms that reconcentrate spots also align them. (a) original spots. (b) Spots developed by PMD. (For other examples, see refs. 11 and 14.)

origin nor with reference to the solvent front, but simply from the edge of the plate. The measurements cited were taken from four plates, two samples per plate (Table 1).

Another test[13] was made using twelve plates with one sample per plate. The samples were deposited as single 5-μl volumes of a solution containing, per μl, 0.2 μg each of amphetamine and methamphetamine. Spot positions were measured, as usual, from the edge of the plate (Table 2).

The solvent in this separation was acetone, whereas in the first example cited it was a mixed solvent, *viz.* ethylene dichloride–ethyl acetate (8:13). The positional

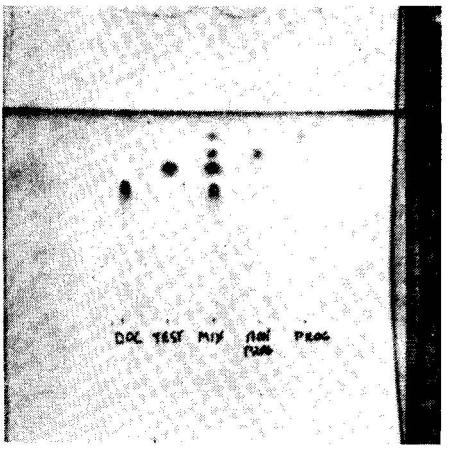

Fig. 5. PMD separation of sterones that requires dried nitrogen for solvent removal. Five cycles in Mode 2 were used, requiring about 41 min overall[18].

TABLE 1

REPRODUCIBILITY OF PMD SPOT POSITIONS USING A MIXED SOLVENT, ETHYLENE DICHLORIDE–ETHYL ACETATE

Compound	*Distance (mm)*		*Relative S.D. (%)*
	Mean	*S.D.*	
DOC	57.0	0.84	1.5
Testosterone	62.0	1.22	2.0
17-OH Pr.	65.1	1.61	2.5
Progesterone	68.8	1.47	2.1

reproducibility of PMD spots does not depend on whether the solvent is simple or mixed.

The positions of PMD spots can be precisely measured not only because the spot locations are highly reproducible but also because the spots themselves are not diffuse. The minimum top-to-bottom "width" of such spots on commercial plates made with silica gel particles of 20–40 μm in diameter is around 1 mm, *i.e.* about 25–50 particle diameters. The usual "working" top-to-bottom spot "width" is less than 2.5 mm[14].

PMD is often applied to improve trace detectability. Fig. 6 shows four chromatograms on one plate, each chromatogram containing 100 ng of estrone and estradiol deposited either from dilute or concentrated solutions. Unlike the ruined conventional chromatogram shown, the PMD chromatogram made from a single deposition of 100 μl of less concentrated solution shows about the same resolution and molecular density per spot as that made from 1 μl of the 100-times more concentrated solution[19].

Fig. 7 shows PMD chromatograms involving origins both contaminated and extended. Made from quinine-spiked raw urine, the origins extended some 20 mm, top to bottom[12]. The developed quinine spots, however, measure about 3 mm top to bottom and allow a good detectability of about 5 μg/μl.

Molecular density, and thus trace detectability, can be still further increased by either narrowed channels or centered PMD. The channels are made about 2 mm wide, rather than narrower, primarily because channels much narrower are hard to make and hard to judge with a pipet. (For very demanding separations and determinations, such narrower channels should be considered.) The channels can be made either from a conventional plate, that is, with the adsorbent bed lying on a supporting plane[11], or with a grooved plate, that is, with the narrow adsorbent beds lying in the grooves[20]. Of the two, the grooves yield the more dense and uniform spots. The

TABLE 2

REPRODUCIBILITY OF PMD SPOT POSITIONS USING A PURE SOLVENT, ACETONE

Compound	*Distance (mm)*		*Relative S.D. (%)*
	Mean	*S.D.*	
Amphetamine	61.6	1.6	2.6
Methamphetamine	41.6	0.6	1.4

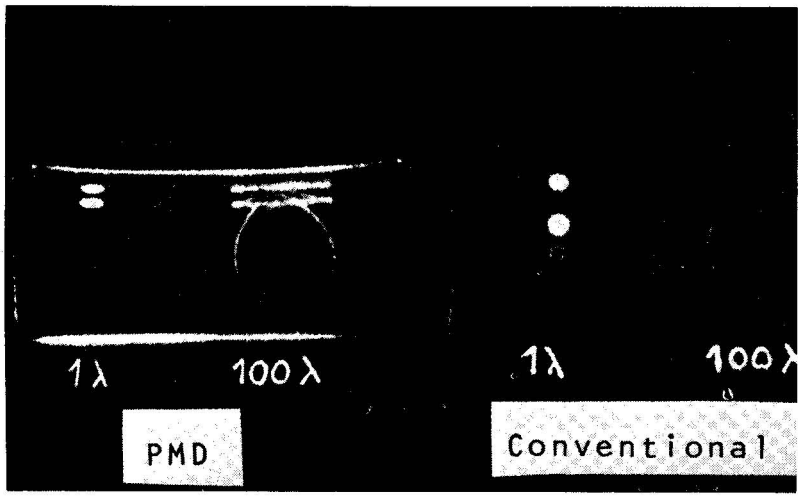

Fig. 6. PMD spots are tight and, within wide limits, do not reflect the characteristics of the origin. Each of the four chromatograms contains 100 ng of estrone and estradiol deposited either from concentrated (left) or dilute (right) solutions[19].

elongated origins resulting from depositing normal microliter volumes of solution on to such channels do not matter with PMD. Sensitivity enhancements of about 50 over conventional TLC are found. Also, many more channels per plate can be accommodated, so that many more samples can be developed simultaneously on one plate.

Another approach that includes higher molecular density among its attributes

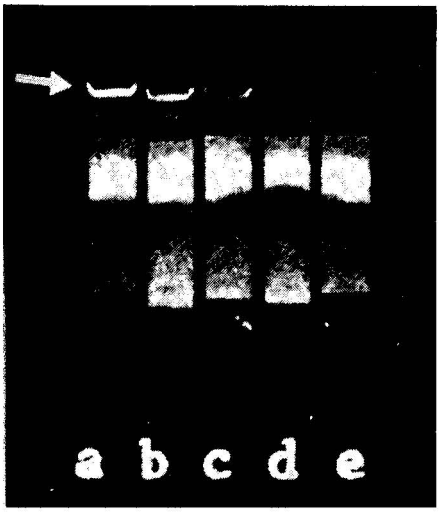

Fig. 7. PMD concentrates traces even from origins that are both broadened and contaminated. Here, quinine traces are retrieved with good sensitivity from 50-μl samples of raw urine[12,14]. Quinine (arrow) concentrations of 5, 2.5, 1.25, 0.625, and 0 μg/ml are shown in chromatograms a through e, respectively. Solvent: acetone. Seven mode 2 cycles.

is centered PMD[16]. Conventional non-channeled TLC plates are used. Evaporation is caused to occur preferentially along the line of the chromatogram. In consequence, the molecules of the solvent and, therefore, of the solutes move toward the center line of the chromatogram from each side. With this technique, both lateral and longitudinal diffusion are countered. As a result, spot molecular density soon reaches a certain level characteristic of the program and of the spot, once it is isolated, and thereafter remains constant. Trace components show spot diameters of 1–2 mm. The chromatograms shown in the Experimental section of this paper were made with centered PMD.

2. BRIEF OUTLINE OF PMD PROGRAMMING AND INSTRUMENTATION

Much more detailed descriptions of PMD programming and instrumentation than this have been published[7,9,14].

PMD solvent advances, initially very short, always increase. The manner of increase depends on the mode.

The duration of the first solvent advance is identical with the unit time for any succeeding solvent advance. Unit times range in 10-sec steps from 0 to 100 sec.

Each solvent advance is followed by a solvent removal. A solvent advance segment followed by a solvent removal segment makes up a cycle.

The segment duration T depends on the mode, the cycle number n, and the unit time t. The maximum cycle number also varies with the mode (Table 3).

TABLE 3

DEPENDENCE OF SEGMENT DURATION (T) ON MODE AND CYCLE NUMBER

Mode	*Maximum cycle number*	*Total segment duration (T)*
1	99	$T = n\,t$
2	28	$T = [n(n + 1)/2]\,t$
3	20	$T = n^2\,t$

The duration of a solvent removal may be fixed or scheduled. If fixed, it is the unit time set for solvent removal, without regard for cycle number. If scheduled, it varies with the mode, cycle number, and solvent removal unit time as just shown.

PMD instrumentation includes primarily a programmer and a developer. The information needed for a PMD program is set into the face of the programmer by the operator. The developer then executes the program under the direction of the programmer.

A given PMD, once started, can be carried out automatically without further attention from the operator. It can also be altered while in progress.

3. PMD VARIABILITY

The durations of most programs to date have roughly equalled the durations of the corresponding conventional TLC separations. In such periods, relatively many

programmed multiple developments can be carried out. These usually suffice because spot reconcentration is so quickly effective. The program used for the PMD chromatogram in Fig. 1, for example, produced five multiple developments and lasted 33 min.

The durations of possible 5-cycle PMD programs, however, range from about 3 min to about 3 h. The durations of all possible PMD programs range from less than 1 min to over ten days.

4. EXTENDED PROGRAMS: PMD CAPABILITY, UMC THEORY, AND TESTS

As far as PMD instrumentation is concerned, programs can as easily have many cycles as few. Also, compared to conventional multiple developments, PMD cycles progress at very high frequency.

The many-cycle, high-frequency capability of PMD, certain related properties of the TLC plate, and the properties of the centered PMD spot are mutually complementary for application to extended, *i.e.* many-cycle, programs.

The conventional TLC plate lends itself well to extended PMD programs in two ways. First, the typical silica gel G precoated plate can easily support any currently programmable number of Mode 1 PMDs. (With a 10-sec unit time, for instance, even the last and farthest solvent advance, the 99th, would take only 990 sec, or about 17 min. Such an advance can almost be accommodated on a 10-cm plate even with solvents such as benzene or acetone, so 20-cm plates are more than adequate.) Secondly, when not overloaded, the current TLC plate can easily display one separated spot every millimeter or so. Both capabilities could, if necessary, easily be improved by using an adsorbent of smaller particle size, a simple change to make.

The centered PMD spot exhibits essentially constant molecular population and density. Such a spot can be moved, without changing its shape or density, for any relevant time and over any relevant distance. (For preparative separations, the whole plate can be used and the centered-spot aspect simply disregarded.)

In sum, thoroughly refining the separation of complex mixtures of similar-R_F components by using large, unprecedented numbers of multiple developments has now become feasible.

Increasing the number of multiple developments, it will be seen, yields the results generally found when chromatographic bed efficiency is sharply improved: two components come from what had been thought to be only one, a trace component emerges from under a major, and so forth.

In addition, there is another effect, predicted by Thoma[3,21] for UMC. It is singular in chromatography.

Consider two solutes mutually similar but not chromatographically identical. Thoma showed that in theory two such solutes can be separated to any desired extent by applying an adequate number of multiple developments.

One envisions a set of trial separations. In the set, the selectivities of both the stationary phase and the solvent are held constant. As solvent strength is reduced in each successive trial, the number of multiple developments in that trial is increased enough to move the component pair, on the average, a fraction $(1 - e^{-1})$ or 0.632 of the distance across the bed of stationary phase.

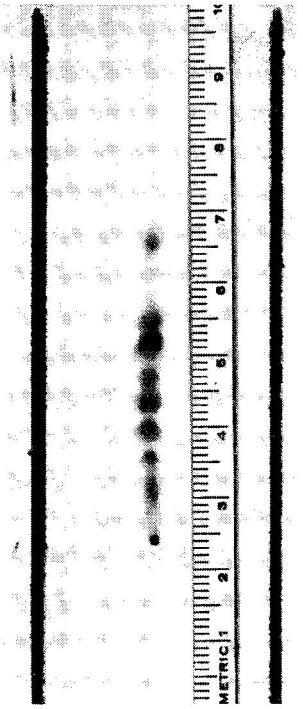

Fig. 8. Centered-PMD chromatogram, 10 cycles, 1.9 h, 1.0-strength dye solution, solvent strength 0.30.

Under this regime, the proportion of the total bed devoted solely to the center-to-center separation of the two components increases with the number of multiple developments required for the 0.632 fractional traverse, and can approach unity. Thus the eventual proportion becomes simply a matter of choice.

The set of related separations shown and discussed in the balance of this paper was prepared as a demonstration and test. We wished to demonstrate: (i) that extended programs are now feasible; (ii) that quite complex mixtures of similar-R_F components can now be cleanly and conveniently separated by TLC in one continuous operation; (iii) that the present conventional TLC plate is adequate for such separations; and (iv) that centered-PMD spots can be moved at will and without spreading over relatively large distances and during relatively long programs. We also wished to test the predictions of the Thoma theory.

5. EXPERIMENTAL

Except for the solvent trough, the equipment, materials and procedures have previously been described[16]. The solvent trough used for the longer developments was fitted with a solvent reservoir arranged to maintain a constant liquid level in the trough.

The development conditions and a guide to Figs. 8–11, as well as the other relevant chromatograms already published[16], are presented in Table 4.

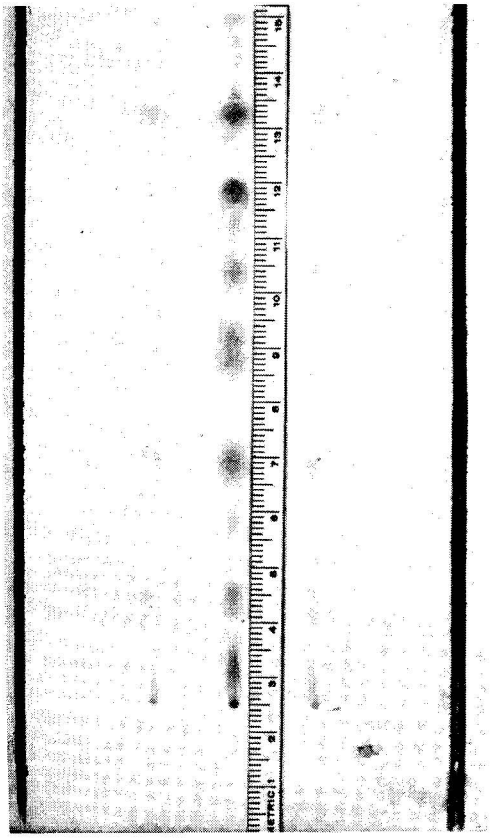

Fig. 9. Centered-PMD chromatograms, 68 cycles, 72 h, solvent strength 0.28. Center chromatogram made from full-strength dye solution, outer chromatograms made from 0.1-strength dye solution.

Figs. 10 and 11 show the five relevant chromatograms redrawn to scale (Fig. 10) and normalized with respect to spot 4 (Fig. 11). In each of these two figures the chromatograms are correlated for spot identification.

The colors and positions of the spots numbered in Figs. 10 and 11 are given in Table 5.

6. RESULTS AND DISCUSSION

The two longest chromatograms —the 32 cycle–17 h chromatogram described earlier[16] and the 68 cycle–72 h chromatogram shown in Fig. 9— were programmed for maximum duration in Mode 1. They establish that such long-lasting programs are feasible. They show that centered-PMD spots do not spread during either movement or duration on the plate. (On the other hand, it should be realized that such numbers of Mode 1 cycles need not take so long. A 32-cycle program can be run in about 2 h, a 68-cycle program in about 9 h.)

Continuously increasing the number of programmed multiple developments

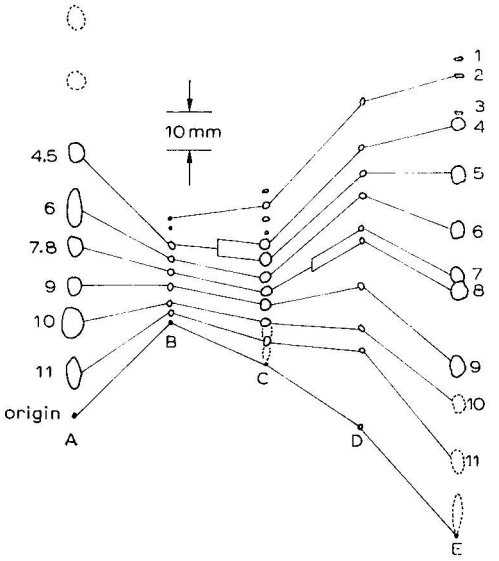

Fig. 10. Test set of extended-program, centered-PMD chromatograms redrawn to scale and displaced vertically for approximate centering.

can continuously improve resolution. Spot [4, 5], for instance, becomes cleanly separated into spots 4 and 5 only after 10 cycles. Also, spot [7, 8] becomes cleanly separated into spots 7 and 8 only after 32 cycles. Spot 4, still exactly superimposed on spot 5 after one development, shows after 68 cycles a center-to-center separation from spot 5 of 14 mm.

Because the successive linear increments of successive Mode 1 solvent advances continually decrease, Mode 1 solvent advance distances increasingly resemble those of UMC. Fig. 11, which presents an approximation to reduced separations (the given

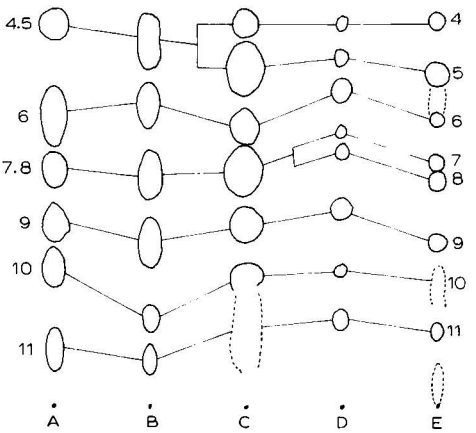

Fig. 11. Test set of extended-program, centered-PMD chromatograms redrawn normalized with respect to spot 4.

TABLE 4

DESCRIPTION OF CHROMATOGRAMS REDRAWN IN FIGS. 10 AND 11

	Chromatogram[a]				
	A	*B*	*C*	*D*	*E*
Figure	—[b]	—[c]	8	—[d]	9
Cycles	1[e]	9[f]	10[g]	32[g]	68[g]
Hours overall	1.3	1.5	1.9	17	72
Dilution spotted[h]	1.0	0.1	1.0	0.1	1.0
Solvent strength[i]	0.32[j]	0.30[k]	0.30[k]	0.30[k]	0.28[k,l]

[a] The chromatograms are redrawn to scale in Fig. 10 and normalized with respect to spot 4 in Fig. 11.

[b] See Fig. 3 in ref. 16.

[c] See Fig. 4 in ref. 16.

[d] See Fig. 5 in ref. 16.

[e] Conventional development.

[f] PMD, Mode 1. Unit times: solvent advance, 100 sec; solvent removal, 100 sec, fixed. Powers: advance, 1; removal, 10.

[g] PMD, Mode 1. Unit times: solvent advance, 100 sec; solvent removal, scheduled, in sec — C, 40; D, 20; E, 30 for 30 cycles, 10 thereafter. Powers: advance, 0; removal, 10. (For explanation of terms, see ref. 16).

[h] One microliter per spot. (For solution composition, see ref. 16).

[i] Solvent strength determined from ref. 24.

[j] Benzene.

[k] Tuned solvents (Regis, Chicago., Ill., U.S.A.).

[l] Tuned solvent mixture, 0.30:0.25, 1:1.

center-to-center separation distances divided by the chromatographic bed length), can therefore be used as an approximate check of the Thoma theory. If the reduced separation for a given spot-pair increases as the number of multiple developments increases, then the lines connecting the spots of that pair should diverge from left to right.

TABLE 5

CORRELATION OF SPOTS SHOWN IN FIGS. 10 AND 11

A–E refer to chromatograms A–E.

Spot No.	*Color*	*Spot position (mm)*				
		A	*B*	*C*	*D*	*E*
1	Yellow					150
2	Maroon		52	66	99	146
3	Gold					136
4	Orange	96	46	55	98	133
5	Light orange	96	46	52	92	119
6	Rose	81	42	47	86	104
7	Coral	70	39	44	77	92
8	Pale coral	70	39	44	74	88
9	Yellow	60	35	40	62	69
10	Gray	50	32	36	51	60?
11	Brown	38	29	31	44	45?
Origin		25	26	25	25	25

(For this use of Fig. 11, however, chromatogram B is not well suited, because the plate was being heated during solvent advance whereas the plates of the other chromatograms were not. Heating the plate compresses the chromatogram. Therefore, in using Fig. 11, chromatogram B should be ignored and instead, the corresponding spots of chromatograms A and C should be mentally connected.)

Chromatograms A (one conventional development) and C (10 cycles) were made with an increasing number of cycles but a decreasing strength of solvents —from 0.32 to 0.30. The (mental) lines from the A-spots to the corresponding C-spots are generally parallel. Thus they show no change in reduced separations.

Chromatograms C (10 cycles) and D (32 cycles) were made with the same solvent strength. Therefore the spots of D should all be displaced toward the solvent front with respect to the spots of C, and the position of increase for the reduced separation should occur at a comparatively low apparent $R_{F,n}$. Spots 5–8 do show the expected displacement toward the front, but more so than does spot 9. Thus the reduced separation between spots 8 and 9 does increase from chromatogram C to D, and the increase corresponds to a comparatively low apparent $R_{F,n}$.

Chromatograms D (32 cycles) and E (68 cycles) were made with an increasing cycle number but a decreasing solvent strength, from 0.30 to 0.28. Therefore, the position of increased reduced separation, if such is to be seen, should be higher in chromatogram E than D. The reduced separation between spots 4 and 5 is indeed seen to increase from chromatogram D to E, and the position of that increase has moved to a higher apparent $R_{F,n}$.

Overall, these observations agree with the predictions of Thoma concerning changes in reduced separation as a function of the number of multiple developments. Increases in reduced separation do occur and do change position as might be expected. However, the increases, which are less than striking, must be sought.

The evidence presented here indicates that any changes in reduced separation produced even by dozens of multiple developments certainly do not dominate the chromatogram. The maximum reduced separation does not by any means usurp the body of the chromatogram, nor do the reduced separations far from the maximum diminish unwontedly.

So long as the chromatogram can be, and is kept, roughly centered on the chromatographic bed by such relatively small alterations in solvent strength as those used here, increasing the number of PMDs merely increases resolution throughout the chromatogram.

Resolution is proportional to the square root of the number of theoretical plates. In conventional TLC, the number of theoretical plates is calculated as $16(x/y)^2$, where x is the distance of the spot from the point of deposition and y is the top-to-bottom spot "width". Because of spot reconcentration, theoretical plates may not be calculated on any multiply developed chromatogram. Spot reconcentration either curtails (UMC) or obliterates (PMD) the normal effects of diffusion. Nevertheless a number of equivalent plates may be calculated from the same measurements and procedure.

We define the number of equivalent plates to be the number of theoretical plates a conventional development would have to produce to generate the spot characteristics being measured. Calculation of the numbers of equivalent plates with these chromatograms throws a new light on the potential of TLC.

The maximum number of theoretical plates attainable in conventional TLC has been discussed by Snyder[22]. For a single 150-mm solvent advance and a spot having the optimal R_F of 0.33, Snyder concluded that 333 theoretical plates could be realized in 30–60 min, or 667 in two directions in 1–2 h. If the spot were carried across the plate by continuous development, as many as 1500 plates could be realized, but 10–20 h would be required.

In comparison, consider spot 2 on chromatogram B[16]. Displaced about 25 mm from the origin and having a "width" of about 1.5 mm, spot 2 shows about 4,500 equivalent plates, produced in 90 min.

(We continue in the next paragraph to cite spot 2 rather than the somewhat more displaced and nominally more favourable spot 1. The presence of spot 1 above spot 2 establishes that spot 2 was not in the solvent front. The top-to-bottom "width" of spot 2 was not adventitious.)

On each chromatogram, spot 2 has a top-to-bottom "width" of 1–2 mm or, as a compromise, 1.5 mm. On chromatogram D, in which it was displaced about 75 mm from the origin, spot 2 shows about 40,000 equivalent plates. On chromatogram E, displaced about 120 mm from the origin, spot 2 shows a little over 100,000 equivalent plates.

These chromatograms were produced on commercial pre-coated plates made with relatively coarse particles that allow faster solvent flow. The minimum spot top-to-bottom "width" found with PMD on such plates equals 25–50 particle diameters[14]. With PMD, as with conventional TLC[22,23], the minimum spot size can be expected to be directly proportional to the particle size. The minimum spot size to be expected on plates made with the 1- to 5-μm-diameter particles known to be more nearly optimum for resolution should be about one-tenth the minimum spot size found here.

A conventional 20-mm TLC plate, made with particles 1–5 μm in diameter and multiply developed with effective R_F-independent spot reconcentration should be able to present over 10^6 equivalent plates throughout the upper half of the plate for spots that are not overloaded.

7. SUMMARY

Introduced in 1951, multiple development by repeated solvent advances improves the resolution available from a given chromatographic system. Repeated solvent advances compress the top-to-bottom "width" of a thin-layer chromatographic spot by the factor $(1 - R_{F,n})$, where $R_{F,n}$ expresses the final location of the spot between the origin and the solvent front. Spots near the front become almost lines drawn parallel to the solvent front, but near the origin spot shapes change little.

In contrast, all spots developed by programmed multiple development (PMD) are line-like. PMD, introduced in 1973, adds solvent removals to solvent advances while, throughout, solvent flows constantly towards the front. Both advances and removals are carried out automatically and relatively rapidly. Used in thin-layer chromatography, PMD spots tend to be uniformly tight and do not reflect the characteristics of the origin. Therefore, PMD is currently used mainly to improve the separability and molecular density of spots.

The form of PMD that is called centered PMD compresses spots laterally as well as longitudinally, so that the molecules of a given spot are brought and held together during successive developments. This further improves trace detectability. It also allows separations to continue without spot spreading as long as necessary or desired.

Thoma predicted that continued multiple development will change the proportion of the chromatographic bed devoted to components ultimately found slightly more than halfway (0.6) toward the solvent front. Tests support the prediction but show that the effect is minor if only some dozens of multiple developments are involved. The principal result of extended programs is a steady improvement of resolution throughout the chromatogram.

REFERENCES

1 A. Jeanes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, 23 (1951) 415.
2 E. Stahl, *Thin-layer Chromatography*, Springer, New York, 2nd ed., 1969.
3 J. A. Thoma, *Anal. Chem.*, 35 (1963) 415.
4 A. S. Saini, *J. Chromatogr.*, 78 (1973) 453.
5 T. H. Jupille and J. A. Perry, *J. Chromatogr.*, 99 (1974) 231.
6 T. H. Jupille and J. A. Perry, *J. Chromatogr. Sci.*, 13 (1975) 163.
7 J. A. Perry, T. H. Jupille and L. J. Glunz, *Separ. Purif. Methods*, 4 (1975) 97.
8 J. A. Perry, T. H. Jupille and A. Curtice, *Separ. Sci.*, 10 (1975) 571.
9 J. A. Perry, K. W. Haag and L. J. Glunz, *J. Chromatogr. Sci.*, 11 (1973) 447.
10 J. A. Perry, T. H. Jupille and L. J. Glunz, *Ind. Res.*, 16 (1974) 55.
11 J. A. Perry and L. J. Glunz, *J. Ass. Offic. Anal. Chem.*, 57 (1974) 832.
12 T. H. Jupille and H. M. McNair, *Amer. Lab.*, 6 (1974) 54.
13 T. H. Jupille and A. Curtice, *Chromatographia*, 8 (1975) 193.
14 J. A. Perry, T. H. Jupille and L. J. Glunz, *Anal. Chem.*, 47 (1975) 65A.
15 J. A. Perry, *US Pat.*, 3 864 250, Feb. 4, 1975.
16 J. A. Perry, *J. Chromatogr.*, 110 (1975) 27.
17 *PMD News*, No. 10, Regis Chemical Company, Morton Grove, Ill.. 1975.
18 *PMD News*. No. 11, Regis Chemical Company, Morton Grove, Ill., 1975.
19 *PMD News*, No. 4, Regis Chemical Company, Morton Grove, Ill., 1974.
20 T. H. Jupille, *26th Pittsburgh Conf. Anal. Chem. Appl. Spectrosc., Pittsburgh, Pa., 1975*, Paper No. 264.
21 J. A. Thoma, *Advan. Chromatogr.*, 6 (1968) 61.
22 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 121.
23 E. Stahl, in K. Macek and I. M. Hais (Editors), *Stationary Phase in Paper and Thin-Layer Chromatography*, Elsevier, Amsterdam, 1965, p. 288.
24 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, pp. 194, 208, 375.

Journal of Chromatography, 113 (1975) 283–302
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CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS

SELECTIVE REVIEW

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CONTENTS

1. INTRODUCTION AND SCOPE OF REVIEW

Optical isomers occur widely in nature and are utilized for pharmaceutical purposes, for experimental biochemical studies, and as intermediates in the syntheses of biologically active products. The physical property of optical rotation is itself used in studies of chemical kinetic mechanisms or metabolic pathways which produce chirality or which involve inversion or racemization in a key step. The importance of methods which distinguish or resolve enantiomers (molecules with a single chiral center) is very great, especially in pharmaceutical applications, where it is well established that different antipodes can have different pharmacological effects[1].

The conventional methods for measuring optical activity are indeed optical, *e.g.* polarimetry, optical rotatory dispersion and circular dichroism. Almost from the first, it was recognized that chromatographic methods could offer distinct advantages in the analysis and separation of optical isomers —providing that such methods could be developed. Among these advantages are: small sample size, independence from the magnitude of specific rotation, and, most important, independence from other optically active species initially present. Chromatographic methods show promise for moderate-scale separations of synthetic intermediates and final products. For large-scale separations and in consideration of the cost of plant-scale resolution processes, extraction and sorption methods offer substantial increases in efficiency over recrystal-

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lization techniques[2]. The latter are much more common because of the limited extent of present knowledge about stereospecific reactions required to tailor such separations.

In recent years, a significant number of advances have been made in chromatographic separation techniques for the resolution of enantiomers, particularly in gas chromatography. In general the resolution of enantiomers by chromatographic means has been achieved by either conversion of the racemate to a mixture of diastereomers by a suitable chemical reaction with a chiral reagent or through the use of a chiral stationary phase. The major emphasis of this review deals with the latter type of separation although the overwhelming majority of successes have been achieved with the former.

2. CONCEPTUAL MODELS FOR RESOLUTION MECHANISMS

The direct chromatographic resolution of enantiomers is complicated by the fact that individual antipodes differ only in their chirality and not in their vapor pressures, solubilities (in achiral solvents), or ionization constants. In gas chromatography, for example, one may write a simplified expression for net retention volume, V'_R, such that $V'_R = p^0\gamma$, where p^0 is the saturation vapor pressure of a solute and γ is the infinite dilution activity coefficient for the solute in a given solvent. Relative retention, α, is then given by $\alpha = (p^0\gamma)_1/(p^0\gamma)_2$ where the subscripts 1 and 2 refer to the two solutes of vapor pressures $p_1{}^0$ and $p_2{}^0$ and to the activity coefficients γ_1 and γ_2 in a given solvent.

In order to effect resolution, either the vapor pressures or the activity coefficients must be different ($\alpha > 1$). In liquid chromatography a loose analogy can be made between vapor pressure as described and solubility. The vapor pressure or solubility can be altered by derivatization of the enantiomers to form diastereomers. In this case it is not unlikely that p^0 (or solubility, in liquid chromatography) as well as γ are changed, but the only practical requirement is that $\alpha > 1$. The more elegant (and the analytically more sound) approach is the use of chiral solvents or sorbents where a diastereomeric complex is formed by solute–solvent interactions not involving the formation of a covalent bond. Here the relative stability difference (or the difference in γ values) is the controlling separation factor.

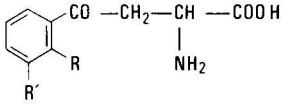
The most commonly used physical picture for this latter type of interactions is one which has its origin in early enzymology and which was adopted by Dalgliesh[3] to explain the retention behavior of amino acid antipodes in cellulose paper chromatography. This model is often termed the "three-point" model because it requires three significant points of attachment between the solute and the sorbent (hydrogen bonds, directed, complexation, etc.). Heavy emphasis has been made in the literature on the need for "attachment" and many molecular parameters have been strained to achieve such bonding schemes. The more adventurous authors suggest a steric interaction as one of the three and use only two points of "attachment". The point missed here is that the statistical requirement of this model is a minimum of three stereochemically significant interactions. For example, three significant finger contacts are required to distinguish a right or left human hand from the "handedness" of ones own probe hand. As the number of interactions multiply, distinction becomes easier and

easier (*e.g.*, entire hand clasp). It is perfectly reasonable then to predict that an environmental chirality would distinguish enantiomers, that is, a solvent or sorbent in (or on) which no "attachments" occur and all of the interactions are steric.

The practical consideration is the magnitude of the difference in the interaction. This is conveniently described as the difference in the thermodynamic standard free energy of formation of the complexes, $\Delta(\Delta G^0)$. If the plate requirement for separation is to remain below 10^5, $\Delta(\Delta G^0)$ must be greater than about 10 cal (equivalent to $\alpha = 1.01$ at 398°K). For preparative-scale, packed column experiments, $\Delta(\Delta G^0)$ values of about 300 cal ($\alpha = 1.5$ at 398°K) are desirable. Therefore, while there is no reason to believe that a difference in interaction energy does not exist for any antipodal racemate in any chiral solvent, only certain solvents (sorbents) will yield practical, useful results.

3. LIQUID MOBILE PHASE–CHIRAL STATIONARY PHASE METHODS FOR DIRECT RESOLUTION

The resolution of racemic amino acids was first reported by Kotake *et al.*[4]. Dalgliesh[5] subsequently studied the structural features necessary for resolution to occur in aromatic amino acids. Three basic structural types were investigated (I–III).



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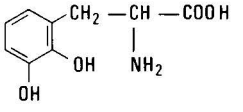

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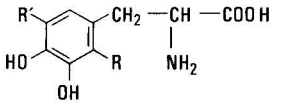


III

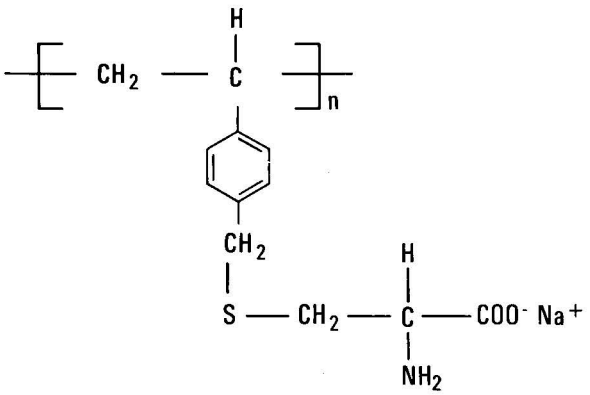
Resolution was observed with (II) and with (III; R = CH_3, R′ = H) but not with (III; R = R′ = H) or with (III; R = H, R′ = CH_3).

Dalgliesh[5] attributed the separation to a differential adsorption phenomenon with "flat surface" molecules (*e.g.*, aromatic) and to hydrogen-bonding molecules being strongly sorbed to the "flat architecture" of cellulose. He proposed that the amino group and possibly the carboxyl group should be "intact". If both these groups were unable to become simultaneously attached to the cellulose surface, presumably by hydrogen bonding, resolution would be unlikely as a three-point attachment of the molecule is required for stereochemical specificity. To give this "three-point attachment", Dalgliesh reasoned that the molecule must contain some other portion, such as an aromatic ring, which is adsorbed on the cellulose surface. In addition, the ring must carry one or more substituents allowing a closer "fit" with the cellulose surface, and hence greater adsorption with one of the optical isomers than with the other. From the work already done, it was postulated that any phenylalanine containing a small substituent in the *ortho* position may be resolvable due to preferential steric effects.

Resolution of the pharmacologically significant *d,l*-DOPA (III; R = R′ = H) was reported by Baczuk *et al.*[6]. The separation was accomplished by using an asymmetric adsorbent (bonded liquid stationary phase) obtained by linking L-arginine via a cyanuric linkage to a commercial polydextran medium conventionally used for exclusion chromatography. An α value of 1.6 was obtained for DOPA and a partial

resolution of tyrosine was observed. Phenylalanine was not resolved. The choice of arginine was based on the need for a three-point contact and for a molecule which could be separated by a bonding link from the surface in such a way that the support backbone did not interfere with the desired "three-point" contact. Interestingly, Bradley *et al.*[7] had postulated in 1954 that the arginine residues in natural wool were the active functions in the optical resolution of mandelic acids which occurs when wool is immersed in an aqueous solution of the racemate salt. In addition, the dextran gel itself used in the former study was reported by Leitch *et al.*[8] to give partial resolution of racemic mandelic acid. (Mandelic acid is used in many studies because of its general solubility and its high specific rotation, $[\alpha]_D^{20} = 158°$, in water.)

There followed a long series of attempts to synthesize polymer resins, wherein an optically active group was introduced into the polymer backbone and which could serve as adsorbents for the resolution of racemic mixtures. Worthy of note is the synthesis by Roberts and Haigh[9] of a crosslinked (1.5% divinylbenzene) poly-[S-(*ar*-vinylbenzyl)-l-cysteine] resin:



R,S-methionine was resolved to a maximum of 44% with this resin using water eluent. Suda *et al.*[10] have reported a new acrylic ion-exchange resin. The resin was prepared by polymerization of ethyl-N-acryloyl-*l*-pyroglutamate and divinylbenzene. This acid-type resin is capable of up to 90% resolution of basic amino acids such as lysine and ornithine. Neutral amino acids are only partially resolved.

Davankov *et al.* have recently reported a technique which they term "ligand-exchange chromatography" (ref. 11 and references therein). The ligand-exchange method differs from adsorption or ion-exchange in that the stationary phase–sorbate interaction is due to the formation of a coordination bond(s) inside the coordination sphere radius of a complexing metal ion. This process, they claim, enhances any sorbent–sorbate interaction which occurs. The complexing metal ion can be bound to mobile ligands and in this case separation is based on the chromatography of several ligand–metal complexes. If the metal ion is bound to the non-mobile ligands of a polymeric stationary phase, then separation arises from chromatography of mobile phase-borne ligands. Rogozhin and Davankov[12] reported separations of a number of racemates (proline for instance) on a chloromethylated styrene–*p*-divinylbenzene copolymer with L-proline.

Humbel *et al.*[13] partially resolved some amino acid derivatives into their stereo-

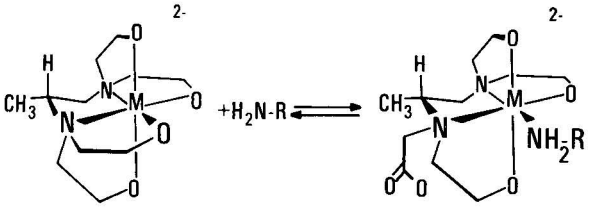
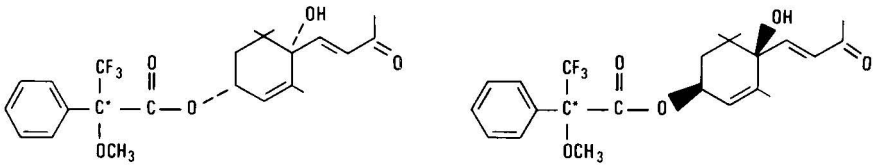


Fig. 1. Replacement of a carboxyl group by an exchange ligand, 1-phenylethylamine, in metal ion propylenediaminetetraacetates.

somers by using an ion-exchange resin containing a chiral anionic iron chelate. Two prerequisites noted for this chromatographic method were: (1) the asymmetric ligand must be labile to allow for ligand exchange and (2) the asymmetric ligand must maintain its asymmetric structure around the metal ion. Bernauer *et al.*[14] examined selectivity in formation of mixed complexes of optically active nickel(II), copper(II), and zinc(II) propylenediaminetetraacetates (PDTA) with 1-phenylethylamine. In the metal ion–PDTA complex, the exchange ligand (1-phenylethylamine) probably replaces a carboxyl group as shown in Fig. 1. Approximately 60% resolution of 1-phenylethylamine was achieved with a short column of Dowex 1-X2 saturated with $[Cu\text{–}D\text{-}PDTA]^{2-}$.

4. LIQUID MOBILE PHASE–ACHIRAL STATIONARY PHASE METHODS FOR INDIRECT RESOLUTION

With proper derivatization enantiomers can be resolved in many cases via diastereomers. A reagent for assessment of optical purity by nuclear magnetic resonance[15] has found use for derivatization for liquid chromatography: (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride. Koreeda *et al.*[16] demonstrated that preparative-scale separation of the enantiomers of abscisic acid was feasible by high-speed liquid chromatography of the acetate esters made from the latter acid and the chiral acid chloride, (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTP ester):



The MTP ester was used for separation of diastereomers by Nakanishi *et al.*[17] in connection with studies of insect juvenile hormone.

Helmchen and Strubert[18] showed that diastereomeric amides formed from reactions of racemic amines with optically pure O-methylmandelyl chloride were generally separable by high-pressure liquid chromatography. This method appeared particularly well suited for detection of trace amounts of optical impurities.

5. INDIRECT RESOLUTION BY GAS CHROMATOGRAPHIC METHODS

Observations which dealt with gas chromatographic resolution of diastereomers began to appear in the literature around 1960. Gil-Av and Nurok[19] completely resolved a series of racemic secondary alcohols as the lactic acid derivatives on a 150-ft. capillary column coated with polypropylene glycol. A similar approach was later used by Gil-Av *et al.*[20] to resolve racemic amino acids. The amino acids were chromatographed as the N-trifluoroacetyl (N-TFA) esters of 2-*n*-alkanols. The latter technique was applied to the problem of the determination of the configuration of the amino acids in two antibiotics of the vernamycin B group[21]. Gil-Av and Nurok[22] have written an extensive review of optical isomer resolution by gas chromatography of diastereomers describing procedures, classes of compounds resolved, applications, and also what is known about the mechanism of resolution.

Considerable advances toward understanding the separation mechanism(s) for diastereomers have been made by Karger and co-workers. In one study[23], diastereomeric esters of α-acetoxypropionic acid were chromatographed at the same temperature on polar and non-polar columns. Greater free energy differences on the polar column indicated larger differences in interaction between the diastereomers and the stationary phase. Also, with increasing size of the substituent at the ester asymmetric carbon, resolution of the diastereomers improved, indicating an assist in asymmetric environment at the central ester linkage due to increased conformational immobility caused by the increased steric bulk. Further experiments indicated that the distance between optical centers in the esters was critical to the separations since resolution was lost as the number of methylene groups between those centers increased.

The concept of conformational immobility previously mentioned (and the consequent nonequivalent accessibility of the ester linkage for interactions of the diastereomers with the stationary phase) was expanded in a further study by Karger *et al.*[24]. A number of racemic cyclic amines were resolved as the N-TFA L-prolyl derivatives. In all cases, both asymmetric carbon atoms in the derivatized compounds were part of cyclic systems. Because free rotations about bonds on the ring are impossible, groups attached to the asymmetric centers are immobile. A very large difference in interaction with the stationary phase was noted for the N-TFA L-prolyl derivatives of 2-methylindoline, due probably to the especially rigid planar arrangement created by the five-membered ring. This greater rigidity leads to increased chromatographic resolution.

N-TFA L-prolyl chloride is a highly versatile reagent for gas chromatographic optical purity analysis of primary and secondary (including cyclic) amines[25–28] and amino acids as their esters[27,29]. However, the proline reagent will not always be significantly more effective than other reagents for resolution of amines. In recent work by Souter[30], several new amino acid chloride resolving agents were compared to the prolyl reagent for resolution of amphetamines and related amines. As shown in Fig. 2, 1-methyl-3-phenylpropylamine was better resolved with N-TFA L-leucyl chloride than with N-TFA L-prolyl chloride. Substituent changes at the chiral centers of the amines, in close proximity to the peptide amide linkage, were shown to affect the resolution. N-Pentafluoropropionyl (N-PFP) and N-heptafluorobutyryl (N-HFB) derivatives were shown in many cases to give nearly the same degree of resolution as in the cases of the N-TFA analogs, but in much less time.

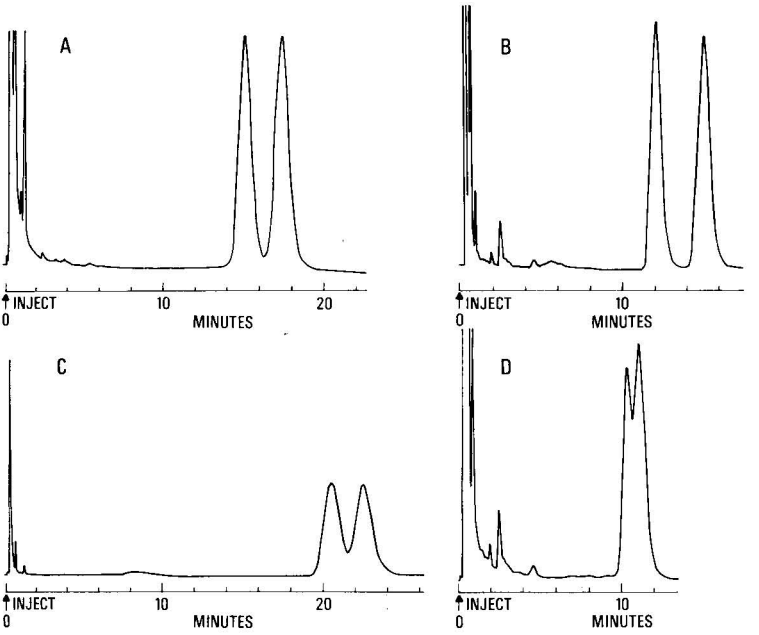


Fig. 2. Comparison of separations of N-TFA L-prolyl and N-TFA L-leucyl amine diastereomers at 200°C on a 6-ft. glass column packed with 5% DEGS on 70–80 mesh Anakrom AB with a flow-rate of 60 ml/min of helium. (A) N-TFA L-leucyl-*d,l*-1-methyl-3-phenylpropylamine, $\alpha = 1.153$. (B) N-TFA L-prolyl-*d,l*-α-ethylphenethylamine, $\alpha = 1.243$. (C) N-TFA L-prolyl-*d,l*-1-methyl-3-phenylpropylamine, $\alpha = 1.096$. (D) N-TFA L-leucyl-*d,l*-α-ethylphenethylamine, $\alpha = 1.078$.

6. DIRECT RESOLUTION BY GAS CHROMATOGRAPHIC METHODS

In early work a mixture of success and failure is apparent and a conflict of results exists[31–33]. The partial resolution of octahedral chromium complexes by gas–solid chromatography using purified helium and powdered "dextro" quartz has been reported but peaks were observed to tail for hours and the extent of resolution was marginal[34].

At the Sixth International Symposium on gas chromatography in Rome, Gil-Av *et al.*[35] reported some preliminary results of optical isomer separation experiments. Eighteen pairs of N-TFA α-amino acid esters were resolved on glass capillary columns coated with N-TFA D- or L-isoleucine lauryl ester and with N-TFA L-phenylalanine cyclohexyl ester. The separation of antipodes was theorized as involving readily reversible association between the enantiomers and the asymmetric solvent molecules. The transient diastereomeric association complexes could have different steric and polar interactions between substituents on asymmetric centers. Up to a limit the increase in bulk of the substituents on the α-carbon and on the ester group was found to enhance resolution. There followed a series of papers which stimulated investigation by several other groups[36–39].

The three basic stationary phase structural types devised by Gil-Av and Feibush are shown in Fig. 3. The amino acid ester-amides (A) and the dipeptide ester-amides have been more extensively studied because of the substantially larger relative

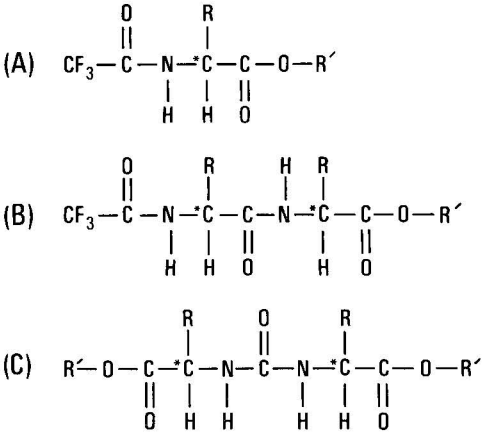


Fig. 3. Basic structural types of optically active stationary phases for gas chromatography.

retention values (α) observed when they are used as liquid phases. The third type (C) is frequently (but erroneously) referred to as a "ureide", but ureides are diacyl ureas and the proper nomenclature here is carbonyl-bis-(amino acid ester). Initial studies of this latter stationary phase type (as liquids) resulted in appreciably smaller α values than A or B, which appeared to limit the utility.

The first dipeptide phase, N-TFA L-valyl-L-valine cyclohexyl ester, produced relative retention values so large as to permit resolution of amino acid enantiomers on a 2-m packed column[40]. This latter result showed that the potential for practical separation of enantiomers by gas chromatography existed. Subsequently, phases like N-TFA L-phenylalanyl-L-leucine cyclohexyl ester were synthesized, which overcame the temperature lability of the first phase[41].

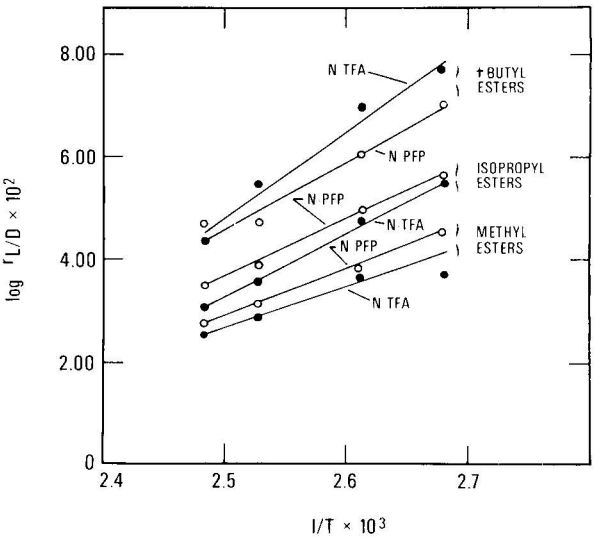


Fig. 4. Plot of the logarithm of the resolution factor r(L/D) *versus* the inverse of the absolute temperature for N-TFA (●) and N-PFP (○) esters of norleucine.

Comparisons of the latter two dipeptide phases showed that resolution improved as the alkyl residue of the solute ester function changed from primary to secondary to tertiary[42]. In addition, the alkyl radical at the solute asymmetric carbon decreased resolution when substituted in the β position, while the reverse was true of substitution in the γ position. Under similar conditions, N-PFP derivatives had about 25% shorter retention times than the corresponding N-TFA esters, with similar relative retentions. Some of these observations are summarized in Fig. 4 for norleucine[42].

A tripeptide phase, N-TFA L-valyl-L-valyl-L-valine isopropyl ester was synthesized by Feibush and Gil-Av[38]. Utility for resolution of optical isomers was good but relative retention values for a variety of solutes were smaller than the analogous dipeptide results.

N-Lauroyl L-valyl-*tert.*-butylamide was reported by Feibush as an asymmetric phase for enantiomer separations[39]. Compared to previously discussed dipeptides the new phase showed increased selectivity and thermal stability, and resolution factors for a number of N-TFA α-amino acid methyl esters were reported to be the largest published for such derivatives on asymmetric phases. These values are shown in Table 1.

Feibush and Gil-Av proposed a theoretical explanation for the resolving power of the dipeptide phases[38]. The fundamental basis is, they state, the formation of transient diastereomeric complexes. These authors argued that such association imposed a particular conformation on the solutes, with the acceptor and donor forming part of a spiral turn. The chirality in each case was determined by the configuration of the amino acids in the dipeptide. The resulting distortion, occurring in the same direction for both enantiomers on the particular stationary phase, had the effect of introducing a conformational element of chirality. In the conformation in which they bonded to the solvent (dipeptide), the enantiomers thus ceased to be mirror images and became "conformational diastereomers". Interactions of such diastereomers with the solvent

TABLE 1

RESOLUTION FACTORS *r*(L/D) OF N-TFA α-AMINO ACID METHYL ESTERS

Column: 150 ft. × 0.02 in. I.D., capillary, coated with N-lauroyl L-valyl-*tert.*-butylamide at 130° (ref. 39).

Amino acid	*r*(L/D)
Alanine	1.188
Valine	1.170
O-TFA threonine	1.117
tert.-Leucine	1.084
Alloisoleucine	1.186
Isoleucine	1.159
Leucine	1.280
Proline	1.057
O-TFA serine	1.101
Aspartic acid	1.078
Glutamic acid	1.170
Methionine	1.215
Phenylalanine	1.198
O-TFA tyrosine	1.262

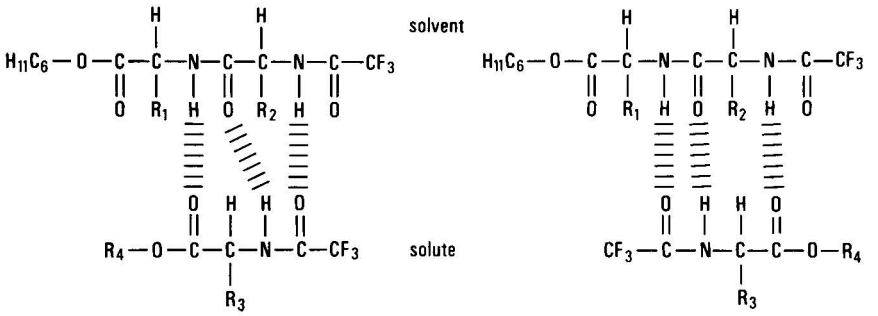


Fig. 5. Possible hydrogen bonding formation of diastereomeric association complexes between N-TFA amino acid ester (solute) and N-TFA dipeptide cyclohexyl ester stationary phase (solvent).

were different and allowed resolution to occur[38]. The asymmetric center substituents of the dipeptide and of the solutes, and the ester substituents of the dipeptides were found to influence the separations. Further understanding of the molecular basis for enantiomer separations is provided by the work of Parr and Howard[43–47] and of Corbin *et al.*[48].

Structural effects in a variety of peptide phases were examined by Parr and Howard, who contend that the amide portion of the stationary phase molecules is the primary contributor to the separations[47]. For association of N-TFA amino acid esters with N-TFA dipeptide cyclohexyl ester phases they have pictured the diastereomeric hydrogen-bonded association complexes similar to Fig. 5. Table 2 presents structures of four dipeptide phases studied recently by Parr and Howard[47]. Separations of a number of N-TFA amino acid isopropyl esters were accomplished using the phases in Table 2. The butyric acid cyclohexyl ester phase was found to effect complete resolution of the amino acids in the mixture tested as well as their respective enantiomers. Increases in the size of alkyl substituents on the asymmetric centers of the dipeptide (solvent) produced greater solute–solvent interactions while similar modifications of the side-chain on the solute α-carbon caused decreased interactions. For dipeptide phases, Parr and Howard found the amide portion of the solvent molecule to be the segment most important in complex formation. All separations were accomplished using extremely long capillary columns.

TABLE 2

HOMOLOGOUS DIPEPTIDE STATIONARY PHASES

$CF_3CO\text{–}NH\text{–}\overset{*}{C}H(R_1)\text{–}CO\text{–}NH\text{–}\overset{*}{C}H(R_2)\text{–}COOC_6H_{11}$

Phase	R_1, R_2
N-TFA L-alanyl-L-alanine cyclohexyl ester	$-CH_3$
N-TFA L-α-amino-*n*-butyryl-L-α-amino-*n*-butyric acid cyclohexyl ester	$-CH_2CH_3$
N-TFA L-norvalyl-L-norvaline cyclohexyl ester	$-CH_2-CH_2-CH_3$
N-TFA L-norleucyl-L-norleucine cyclohexyl ester	$-CH_2-CH_2-CH_2-CH_3$

Corbin *et al.*[48] have also examined effects on gas chromatographic separations due to systematic structural variations in the peptide phases. Their study also indicated that the amide end of the dipeptide appeared to make the primary contribution to the separations, although resolution was sensitive to changes at the ester end. Steadily poorer resolution resulted from increasing the bulkiness of the peptide side groups. These authors also examined a tripeptide phase and found essentially the same relative retention values as yielded by the dipeptide. This latter observation was significant in that it indicated that the ester and amide portions need not be close to each other for resolution to occur. In addition, advantages of N-PFP stationary phases over N-TFA phases were apparent.

The other major class of optically active stationary phase useful for direct gas chromatographic enantiomer separations is the carbonyl-bis-(amino acid ester), the first example of which was reported by Feibush and Gil-Av[36]. The fundamental nature of solvent–solute interactions for this phase type was studied by Corbin and Rogers[49] and to a larger extent by Lochmüller *et al.*[50–54].

The initial impetus for the preparation of carbonyl-bis-(L-valine isopropyl ester) was to allow more equivalent hydrogen-bonded association interactions in close proximity to asymmetric carbons. This phase was demonstrated to have a significant resolving power for chiral primary amines as N-TFA derivatives[36].

Corbin and Rogers conducted a systematic investigation of the chromatographic properties of this phase[49]. Successful resolution of secondary amine derivatives on the liquid phase was reported using capillary columns, and factors affecting separations such as temperature, per cent loading of the column, sample size, and flow-rate were examined. It was found that not only would the phase as a liquid yield enantiomer separations, but that when operated below its melting point the phase gave greatly enhanced separations. The authors suggested that the enhanced activity results from the increased structural rigidity of the solid phase which causes a more fixed interaction geometry than is available with the liquid phase[49]. Peak shapes were anomalous in that the leading peak was sharp while the peak for the longer-retained enantiomer was very broad; on the liquid phase, both peaks for any particular separation were essentially identical.

Lochmüller *et al.* initially reported NMR studies of the sites of hydrogen bond formation with two carbonyl-bis-(amino acid esters) and various solutes[50]. Association chemical shifts with carbon tetrachloride as the solvent medium showed (by ^{13}C and by ^{1}H NMR) that the only significant hydrogen-bonding interaction occurred between the N–H portion of the amide solute and the ester carbonyl of the carbonyl-bis-(amino acid ester). This spectroscopic evidence was interpreted to indicate that only one significant point of "attachment" is involved in formation of the diastereomeric association complexes.

The effect of substituent changes on the donor or acceptor strength of functional groups is also important in determination of the strength of hydrogen bonding, and in the case of the carbonyl-bis-(amino acid esters) the effect on the ester carbonyl of changing the ester substituent from methyl to ethyl to isopropyl and finally to *tert.*-butyl was spectroscopically shown to be inductive in nature[55]. Fig. 6 shows the structures of the carbonyl-bis-(amino acid esters) examined by Lochmüller and Souter[53–55] (spectroscopically and chromatographically). The effect of the bond polarity change on the α-values is complex, with the α-values rising from methyl to ethyl, remaining

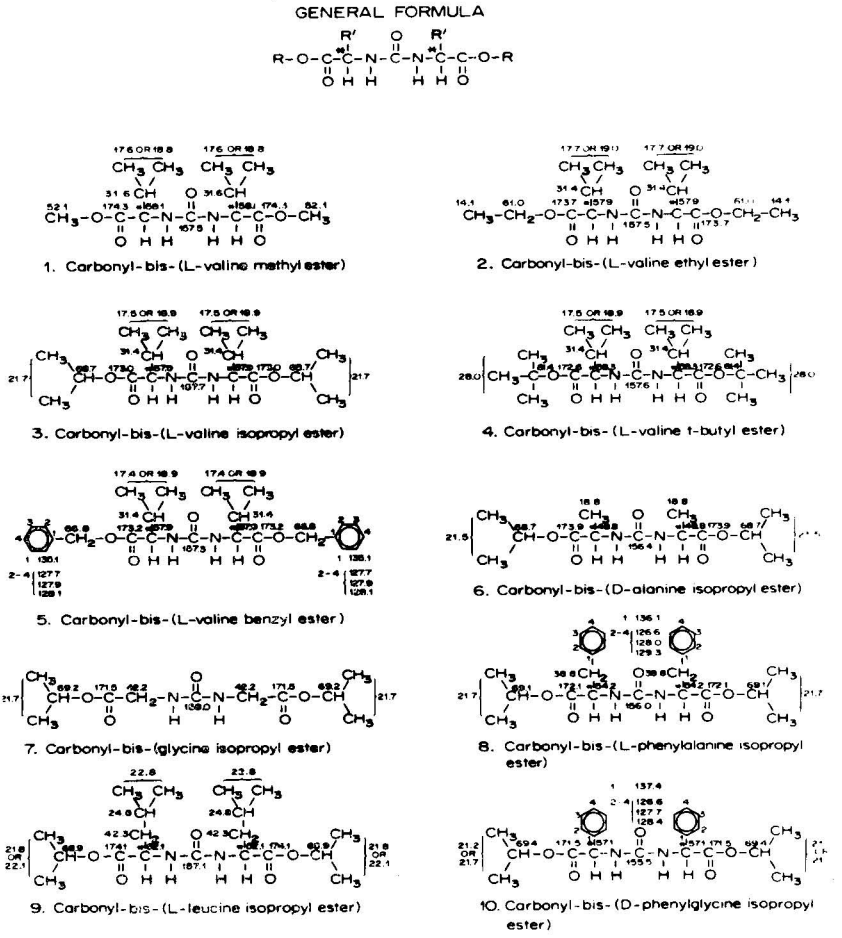


Fig. 6. Structures of chiral (except 7) carbonyl-bis-(amino acid esters). The numerical values are ^{13}C NMR chemical shifts in ppm *vs.* internal tetramethylsilane.

nearly constant at isopropyl, and then decreasing at *tert.*-butyl, as shown in Table 3. The decrease at *tert.*-butyl was attributed to steric hindrance of the large ester group in limiting the approach of hydrogen-bonding donor solutes to the ester carbonyl.

Although it has been suggested that optically active liquid crystal phases should be capable of resolving optical isomers by gas chromatography, such separations were not reported. Kelker and Winterscheidt[56] and Kelker and Von Schivizhoffen[57] have reviewed the use of liquid crystals as stationary phases in gas chromatography and have discussed the physical properties which affect their suitability for such use. Results of numerous gas chromatography experiments with nematic and smectic liquid crystal stationary phases to separate benzene positional isomers have been presented[58,59] with arguments that both the relatively high viscosity and the high degree of order of smectic phases may influence the separations. Cholesteryl ester liquid crystalline phases have also received attention as gas chromatographic stationary

TABLE 3

VARIATION OF α WITH ESTER SUBSTITUENT STRUCTURE OF STATIONARY PHASE FOR ACYLATED α-METHYLBENZYLAMINE SOLUTES

Stationary phases as isotropic liquids.

Compound	α
Carbonyl-bis-(L-valine methyl ester)	
N-TFA α-methylbenzylamine	1.065
N-PFP α-methylbenzylamine	1.060
N-HFB α-methylbenzylamine	1.066
Carbonyl-bis-(L-valine ethyl ester)	
N-TFA α-methylbenzylamine	1.096
N-PFP α-methylbenzylamine	1.106
N-HFB α-methylbenzylamine	1.105
Carbonyl-bis-(L-valine isopropyl ester)	
N-TFA α-methylbenzylamine	1.099
N-PFP α-methylbenzylamine	1.100
N-HFB α-methylbenzylamine	1.108
Carbonyl-bis-(L-valine *tert.*-butyl ester)	
N-TFA α-methylbenzylamine	1.017

phases[60] and plots of log retention time as a function of inverse absolute temperature exhibited sharp breaks or slope changes at or near mesophase transitions.

The discovery that enantiomers could be resolved with very large α values on a smectic liquid crystal phase of carbonyl-bis-(D-leucine isopropyl ester) was recently reported by Lochmüller and Souter[53]. In addition, those authors showed that several carbonyl-bis-(L-valine esters) displayed liquid crystalline behavior, and were useful as optically active liquid crystalline gas chromatographic phases[54].

That these chiral phases were indeed liquid crystalline was demonstrated by

TABLE 4

THERMAL TRANSITION PROPERTIES OF CARBONYL-BIS-(AMINO ACID ESTERS)

Compound	*Transition temperature (°K)**	*Transition heat, q (kcal/mole)***	*Transition entropy, ΔS (cal/mole·°K)*	*Total ΔS for $S \rightarrow I$ (%)*
Carbonyl-bis-(L-valine	382 C → S	0.73	1.91	69
methyl ester)	415 S → I	1.73	4.17	
Carbonyl-bis-(L-valine	361 C → S	0.44	1.21	81
ethyl ester)	388 S → I	2.00	5.15	
Carbonyl-bis-(L-valine	364 C → S	2.25	6.18	
isopropyl ester)	372 S → S′	0.10	0.27	38
	382 S′ → I	1.55	4.06	
Carbonyl-bis-(L-valine	398 S → C	1.54	3.87	68
tert.-butyl ester)***	402 I → S	3.27	8.13	
Carbonyl-bis-(D-leucine	328 C → S	5.38	16.4	7.9
isopropyl ester)	383 S → I	0.54	1.40	

* C = Crystal, S = smectic, I = isotropic.

** Heats of transition calculated based on the H_f of indium, 780 cal/mole.

*** Results based on cooling curves: heating produced only one broad transition at 427°K. The reported temperatures are transformation temperatures.

differential scanning calorimetry measurements for which data are shown in Table 4. Per cent of total transition entropy due to the transition to isotropic liquid was calculated since it offers a criterion for deciding whether a transition is cholesteric→isotropic, nematic→isotropic, or smectic→isotropic[61]. A very large percentage is characteristic of a smectic→isotropic transition. The temperature range over which mesomorphic behavior was observed decreased as the size of the ester group increased in the L-valine ester series. Also, the change at the chiral centers from the isopropyl function of valine to the isobutyl of leucine radically changed the observed thermal behavior.

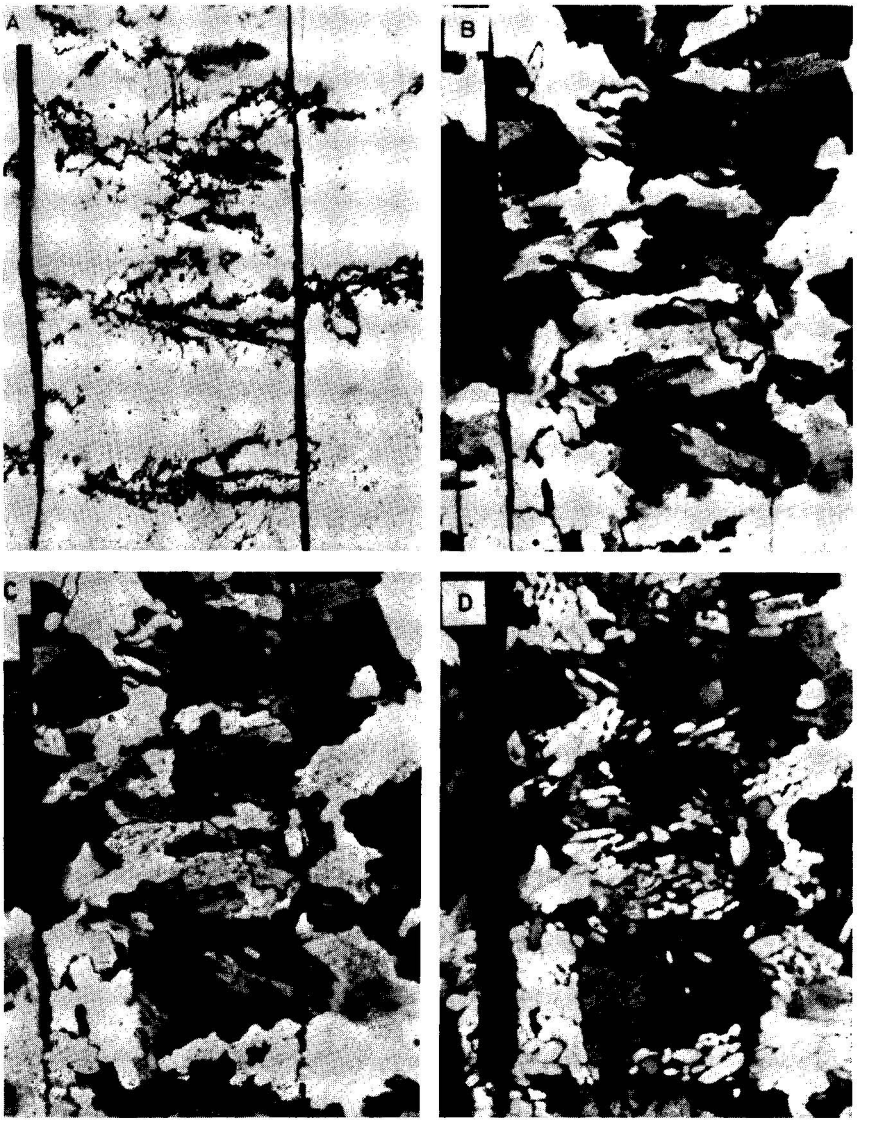


Fig. 7. Polarized photomicrographs of carbonyl-bis-(L-valine isopropyl ester). (A) Crystalline solid; (B) first smectic phase; (C) second smectic phase; (D) changing to isotropic liquid.

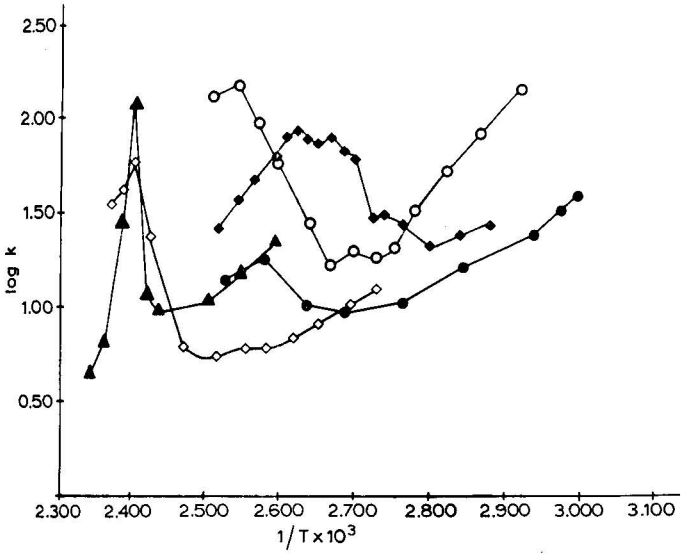


Fig. 8. Plots of log capacity ratio (k) *vs.* inverse absolute temperature for solutes on optically active stationary phases. ▲, N-TFA *d,l*-2-aminopropylbenzene on a 5.0% carbonyl-bis-(L-valine *tert.*-butyl ester) column; ○, N-TFA *d,l*-2-aminooctane on a 5.0% carbonyl-bis-(L-valine ethyl ester) column; ◇, N-TFA *d,l*-2-aminooctane on a 5.0% carbonyl-bis-(L-valine methyl ester) column; ●, N-TFA *d,l*-2-aminooctane on a 5.0% carbonyl-bis-(D-leucine isopropyl ester) column; ◆, N-TFA *d,l*-2-aminooctane on a 1.0% carbonyl-bis-(L-valine isopropyl ester) column. On the carbonyl-bis-(L-valine ethyl ester) phase, N-TFA *d,l*-2-aminooctane showed resolution at some of the temperatures studied; the retention behavior of the leading peak was studied in all cases.

Mesomorphic behavior can also be characterized by microscopy, as was shown by Lochmüller and Souter[51] for phase transitions in carbonyl-bis-(L-valine isopropyl ester), as shown in Fig. 7. Textural and color changes were observable under polarized light as the sample was heated through its phase changes to isotropic liquid.

Phase transitions for the carbonyl-bis-(amino acid esters) could also be determined from plots of the log of the capacity ratio (k) for various solutes *versus* inverse absolute temperature (see Fig. 8). Largest increases in capacity ratios occurred at temperatures where the stationary phases changed to isotropic liquids. When the temperature was increased beyond the isotropic transition point, a simple boiling point relationship was observed in all cases. Relative retentions (α) on the isotropic liquid phases showed significant decreases compared with their values on the smectic phases. Transition temperatures determined by this method agreed closely with those calorimetrically determined.

Carbonyl-bis-(L-valine isopropyl ester), the compound studied by Corbin and Rogers[49] as a solid stationary phase, exhibits two stable smectic states prior to melting. Lochmüller and Souter studied the chromatographic behavior of this and other closely related stationary phases[53,54]. Resolution was achieved in all cases on short packed columns, and the resolving power of the smectic phases was found generally superior to that of the "liquid" state. The largest relative retentions (α) thus far observed for direct gas chromatographic enantiomer separations were reported[54]. Typical results are shown in Tables 5–9. These constitute the first deliberate suc-

TABLE 5

GC SEPARATIONS OF VARIOUS SOLUTES ON A 6-ft. × 1/8-in.-I.D. COLUMN PACKED WITH 5.0% CARBONYL-BIS-(L-VALINE METHYL ESTER) ON 100–120 MESH CHROMOSORB G AW DMCS

Racemic solute	α	$\Delta(\Delta G°)$ *(cal/mole)*
Temperature = 390.19°K (smectic phase)		
N-TFA 2-aminooctane	unresolved	—
N-TFA α-methylbenzylamine	1.062	−46
N-PFP α-methylbenzylamine	1.067	−50
N-HFB α-methylbenzylamine	1.063	−47
Temperature = 415.70°K (isotropic liquid)		
N-TFA 2-aminooctane	unresolved	—
N-TFA α-methylbenzylamine	1.067	−50
N-PFP α-methylbenzylamine	1.072	−58
N-HFB α-methylbenzylamine	1.067	−50

TABLE 6

GC SEPARATIONS OF VARIOUS SOLUTES ON A 6-ft. × 1/8-in.-I.D. COLUMN PACKED WITH 5.0% CARBONYL-BIS-(L-VALINE ETHYL ESTER) ON 100–120 MESH CHROMOSORB G AW DMCS

Racemic solute	α	$\Delta(\Delta G°)$ *(cal/mole)*
Temperature = 385.02°K (smectic phase)		
N-TFA α-methylbenzylamine	1.173	−122
N-PFP α-methylbenzylamine	1.257	−175
N-HFB α-methylbenzylamine	1.336	−222
N-TFA 2-amino-3-phenylpropane	unresolved	—
N-TFA 2-amino-4-phenylbutane	1.108	− 78
Temperature = 398.20°K (isotropic liquid)		
N-TFA α-methylbenzylamine	1.092	− 69
N-PFP α-methylbenzylamine	1.096	− 73
N-HFB α-methylbenzylamine	1.098	− 74
N-TFA 2-amino-3-phenylpropane	unresolved	—
N-TFA 3-amino-4-phenylbutane	unresolved	—

TABLE 7

GC RESULTS OF SEPARATIONS OF SOME PERFLUOROACYL α-METHYLBENZYLAMINES ON A 6-ft. × 1/8-in.-I.D. COLUMN PACKED WITH 5.0% CARBONYL-BIS-(L-VALINE ISOPROPYL ESTER) ON 100–120 MESH CHROMOSORB G AW DMCS (AS A FUNCTION OF TEMPERATURE)

Temperature (°K)		*N-TFA*		*N-PFP*		*N-HFB*	
		α	$\Delta(\Delta G°)$ *(cal/mole)*	α	$\Delta(\Delta G°)$ *(cal/mole)*	α	$\Delta(\Delta G°)$ *(cal/mole)*
Smectic	366.17	1.171	−115	1.366	−227	1.580	−333
	370.05	1.420	−258	1.861	−457	2.205	−581
Smectic	373.34	1.317	−205	1.708	−397	2.116	−556
	377.28	1.242	−162	1.555	−331	1.830	−453
	380.96	1.161	−113	1.340	−221	1.456	−285
Isotropic	396.96	1.112	− 84	1.117	− 87	1.131	− 97

TABLE 8

GC SEPARATIONS OF VARIOUS SOLUTES ON A 6-ft. × 1/8-in.-I.D. COLUMN PACKED WITH 5.0% CARBONYL-BIS-(L-VALINE *tert.*-BUTYL ESTER) ON 100–120 MESH CHROMOSORB G AW DMCS AT 415.61°K

Racemic solute	α	$\Delta(\Delta G^\circ)$ *(cal/mole)*
N-TFA α-methylbenzylamine	1.102	−81
N-PFP α-methylbenzylamine	1.118	−92
N-HFB α-methylbenzylamine	1.099	−78

cessful uses of chiral mesophases for this purpose. Table 10 presents a comparison of separations of N-PFP α-methylbenzylamine enantiomers on columns coated with two different percentage loadings of the valine isopropyl ester phase. It appears that there is a strong dependence of α on the per cent stationary phase coating when the compound is in a smectic state, and a much smaller dependence when it is an isotropic liquid[54].

Retention times of solutes were found to have a definite dependence on sample size for separations on the smectic mesophases. Large samples tended to significantly increase retention time, although the effect on relative retention (α) was small.

The texture of the smectic phases studied is affected by sudden pressure changes since a sudden change in inlet pressure (to achieve higher flow-rates) was seen to reduce α to essentially zero. A substantial time was required before α was restored to its original value at the higher flow-rate.

The most anomalous feature of these separations is the relation of theoretical plate heights in the zones of R and S solutes. The anomalous peak shapes first observed by Corbin and Rogers[49] for enantiomer separations on carbonyl-bis-(L-valine isopropyl ester) as a "solid" phase were also observed by Lochmüller and Souter for separations on that phase as a smectic liquid crystal[54]. A typical chromatogram is shown in Fig. 9. In all cases the second enantiomer to elute showed a significant increase in plate height (fewer plates, larger variance). In general, one would expect that two so closely related species, eluting with relatively large k values and a small α, would have essentially the same plate height.

TABLE 9

GC SEPARATIONS OF VARIOUS SOLUTES ON A 6-ft. × 1/8-in.-I.D. COLUMN PACKED WITH 5.0% CARBONYL-BIS-(D-LEUCINE ISOPROPYL ESTER) ON 100–120 MESH CHROMOSORB G AW DMCS (AS A FUNCTION OF TEMPERATURE)

Racemic solute	*Temperature (°K)*	α	$\Delta(\Delta G^\circ)$ *(cal/mole)*
N-TFA α-methylbenzylamine	361.67	1.119	− 81
N-PFP α-methylbenzylamine	(smectic)	1.658	−363
N-HFB α-methylbenzylamine		1.399	−241
N-TFA α-methylbenzylamine	395.63	1.073	− 55
N-PFP α-methylbenzylamine	(isotropic)	1.080	− 61
N-HFB α-methylbenzylamine		1.079	− 60
N-TFA 2-amino-3-phenylpropane		unresolved	—
N-TFA 3-amino-4-phenylbutane		1.050	− 38

TABLE 10

EFFECT OF PERCENTAGE STATIONARY PHASE LOADING ON SEPARATIONS USING CARBONYL-BIS-(L-VALINE ISOPROPYL ESTER) COATED ON 100–120 MESH CHROMOSORB G AW DMCS, PACKED INTO 6-ft. × 1/8-in.-I.D. COLUMNS

The solute is N-PFP *d,l*-α-methylbenzylamine.

1.0% coating		*5.0% coating*	
Temperature (°K)	α	*Temperature (°K)*	α
380.82 (smectic)	1.116	380.96 (smectic)	1.355
385.28 (isotropic)	1.103	385.28 (isotropic)	1.125
389.42 (isotropic)	1.101	396.96 (isotropic)	1.117

Two possible explanations for this behavior are: (1) mixed isotherm retention mechanisms (liquid surface adsorption, bulk solution and support sorption) or (2) differences in diffusion coefficients for R and S solutes in an ordered chiral liquid crystal solvent. The diffusion rate of a right-handed species in a left-handed matrix could conceivably be different from that of a left-handed species in the same matrix[54].

A major observation to be made based on these results is that the smectic mesophase forms of the optically active stationary phases have a generally greater selectivity towards solutes studied than do the corresponding isotropic liquids. Selectivity increased as the size of the L-valine ester group was increased in that series until *tert.*-butyl. Comparison of results from separations on the L-valine isopropyl ester and the D-leucine isopropyl ester phases shows (besides opposite retention orders due to opposite configurations of chiral centers) that the leucine compound, which bears isobutyl groups at the asymmetric centers as opposed to isopropyl groups in the valine cases, is a qualitatively poorer separator[54]. Selectivity has been shown to be dependent on the asymmetric center substituents, which are in close proximity to the hydrogen bonds to the ester carbonyls[50].

Relative retentions and the differences in standard free energies of association of enantiomers separated on the valine ethyl and isopropyl ester smectic mesophases were very large, whereas in the methyl and *tert.*-butyl cases the measured values were low due perhaps in the former case to the small size of the methyl group and in the latter case to so great a bulk as to hinder hydrogen bonding.

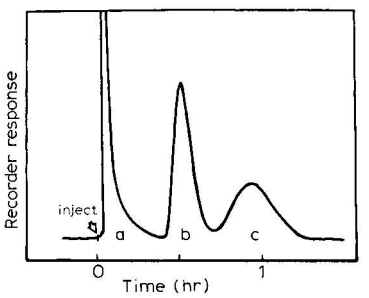


Fig. 9. Anomalous peak shapes observed for N-PFP α-methylbenzylamine enantiomers on a 5.0% carbonyl-bis-(L-valine isopropyl ester) column at 96.89°C (first smectic phase). a = Solvent peak; b = peak for the S solute; c = peak for the R solute.

7. SUMMARY

Recent research has produced a notable increase in knowledge useful for chromatographic enantiomer resolution. Some major advances have emerged in the understanding of asymmetric solute–solvent interactions, and many successful separations by gas and by liquid chromatography have been reported. This review presents a selective discussion of the major advances, with primary emphasis on the use of chiral stationary phases. The latter offer advantages (over indirect techniques) such as generally easier sample preparation, decreased analysis time, and simultaneous chemical as well as optical purity analysis.

REFERENCES

1 E. Costa and S. Garratini (Editors), *Int. Symp. Amphetamines and Related Compounds*, Raven Press, New York 1970, p. 49.
2 D. Buss and T. Vermuelen, *Ind. Eng. Chem.*, 60 (1968) 12.
3 C. E. Dalgliesh, *J. Chem. Soc.*, (1952) 137
4 M. Kotake, T. Sakan, N. Nakamura and S. Senoh, *J. Amer. Chem. Soc.*, 73 (1951) 2973.
5 C. E. Dalgliesh, *J. Chem. Soc.*, (1952) 3940.
6 R. J. Baczuk, G. K. Landram, R. J. DuBois and H. C. Dehm, *J. Chromatogr.*, 60 (1971) 351.
7 W. Bradley, R. Brindley and G. Easty, *Disc. Faraday Soc.*, 16 (1954) 153.
8 R. E. Leitch, H. L. Rothbart and Wm. Rieman, III, *J. Chromatogr.*, 28 (1967) 132.
9 C. Roberts and D. Haigh, *J. Org. Chem.*, 27 (1962) 3375.
10 H. Suda, Y. Hosono, Y. Hosokawa and Y. Seto, *Kogyo Kagaku Zasshi*, 73 (1970) 1250.
11 V. A. Davankov, S. V. Rogozhin, A. V. Semechkin, V. A. Baranov and G. S. Sannikova, *J. Chromatogr.*, 93 (1974) 363.
12 S. V. Rogozhin and V. A. Davankov, *Chem. Commun.*, (1971) 490.
13 F. Humbel, D. Vonderschmitt and K. Bernauer, *Helv. Chim. Acta*, 53 (1970) 1983.
14 K. Bernauer, M. Jeanneret and D. Vonderschmitt, *Helv. Chim. Acta*, 54 (1971) 297.
15 J. A. Dale, D. L. Dull and H. S. Mosher, *J. Org. Chem.*, 34 (1969) 2543.
16 M. Koreeda, G. Weiss and K. Nakanishi, *J. Amer. Chem. Soc.*, 95 (1973) 239.
17 K. Nakanishi, D. Schooley, M. Koreeda and J. Dillon, *Chem. Commun.*, (1971) 1235.
18 G. Helmchen and W. Strubert, *Chromatographia*, 7 (1974) 713.
19 E. Gil-Av and D. Nurok, *Proc. Chem. Soc.*, (1962) 146.
20 E. Gil-Av, R. Charles-Sigler and G. Fischer, *J. Chromatogr.*, 17 (1965) 408.
21 R. Charles-Sigler and E. Gil-Av, *Tetrahedron Lett.*, 35 (1966) 4231.
22 E. Gil-Av and D. Nurok, *Advan. Chromatogr.*, 10 (1974) 99.
23 H. C. Rose, R. L. Stern and B. L. Karger, *Anal. Chem.*, 38 (1966) 469.
24 B. L. Karger, R. L. Stern, W. Keane, B. Halpern and J. Westley, *Anal. Chem.*, 39 (1967) 229.
25 D. A. Mitchard, *J. Org. Chem.*, 34 (1969) 2787.
26 C. E. Wells, *J. Ass. Offic. Anal. Chem.*, 53 (1970) 113.
27 J. Westley, B. Halpern and B. Karger, *Anal. Chem.*, 40 (1968) 2046.
28 E. Gordis, *Biochem. Pharmacol.*, 15 (1966) 2124.
29 J. C. Dabrowiak and D. W. Cooke, *Anal. Chem.*, 43 (1971) 791.
30 R. W. Souter, *J. Chromatogr.*, 108 (1975) 265.
31 G. Karagounis and E. Lippold, *Naturwissenschaften*, 46 (1959) 145.
32 G. Karagounis and E. Lemperle, *Z. Anal. Chem.*, 189 (1962) 131.
33 G. Goldberg and W. Ross, *Chem. Ind. (London)*, (1962) 657.
34 R. Sievers, R. Moshier and M. Morris, *Inorg. Chem.*, 1 (1962) 966.
35 E. Gil-Av, B. Feibush and R. Charles-Sigler, in A. B. Littlewood (Editor), *Gas Chromatography 1966*, Institute of Petroleum, London, 1967, pp. 227–239.
36 B. Feibush and E. Gil-Av, *J. Gas Chromatogr.*, 5 (1967) 257.
37 B. Feibush, E. Gil-Av and T. Tamari, *Isr. J. Chem.*, 8 (1970) 50p.
38 B. Feibush and E. Gil-Av, *Tetrahedron*, 26 (1970) 1361.

39 B. Feibush, *Chem. Commun.*, (1971) 544.
40 S. Nakaparksin, P. Birrell, E. Gil-Av and J. Oro, *J. Chromatogr. Sci.*, 8 (1970) 177.
41 W. Koenig, W. Parr, H. Lichtenstein, E. Bayer and J. Oro, *J. Chromatogr. Sci.*, 8 (1970) 183.
42 W. Parr, C. Yang and E. Bayer, *J. Chromatogr. Sci.*, 8 (1970) 591.
43 W. Parr and P. Y. Howard, *Chromatographia*, 4 (1971) 162.
44 W. Parr and P. Y. Howard, *J. Chromatogr.*, 67 (1972) 227.
45 W. Parr and P. Y. Howard, *J. Chromatogr.*, 66 (1972) 141.
46 W. Parr and P. Y. Howard, *J. Chromatogr.*, 71 (1972) 193.
47 W. Parr and P. Y. Howard, *Anal. Chem.*, 45 (1973) 711.
48 J. Corbin, J. Rhoad and L. Rogers, *Anal. Chem.*, 43 (1971) 327.
49 J. Corbin and L. Rogers, *Anal. Chem.*, 42 (1970) 974.
50 C. H. Lochmüller, J. M. Harris and R. W. Souter, *J. Chromatogr.*, 71 (1972) 405.
51 C. H. Lochmüller and R. W. Souter, *J. Phys. Chem.*, 77 (1973) 3016.
52 C. H. Lochmüller and R. W. Souter, *Amer. Lab.*, 11 (1973) 25.
53 C. H. Lochmüller and R. W. Souter, *J. Chromatogr.*, 87 (1973) 243.
54 C. H. Lochmüller and R. W. Souter, *J. Chromatogr.*, 88 (1974) 41.
55 C. H. Lochmüller and R. W. Souter, *Org. Mag. Res.*, 5 (1973) 373.
56 H. Kelker and H. Winterscheidt, *Z. Anal. Chem.*, 220 (1966) 1.
57 H. Kelker and E. von Schivizhoffen, *Advan. Chromatogr.*, 6 (1968) 247.
58 M. Dewar and J. Schroeder, *J. Amer. Chem. Soc.*, 86 (1964) 5235.
59 M. Dewar and J. Schroeder, *J. Org. Chem.*, 30 (1965) 3485.
60 E. M. Barrall, II, R. S. Porter and J. F. Johnson, *J. Chromatogr.*, 21 (1966) 392.
61 R. Porter, E. Barrall, II and J. Johnson, *Acc. Chem. Res.*, 2 (1969) 53.

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CHREV. 90

CHEMICAL DERIVATIZATION IN GAS CHROMATOGRAPHY

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CONTENTS

1. INTRODUCTION

Gas chromatography (GC) is suitable for the separation and analysis of substances that display an adequate volatility in the chromatographic system used. The criteria for this general definition are rather loose, as there are a number of ways in which the volatility of a given solute component can be controlled. These aspects have been studied since the advent of GC and, in many respects, it was these problems that led to new concepts in GC techniques and instrumentation and to the development of new chromatographic materials. Examples are high-temperature GC, temperature programming, the use of sorbents of high selectivity, operation in systems with a low content of the sorbent, high-pressure and supercritical-fluid chromatography (up to the transition to liquid chromatography) and chemical conversion of the substances to be chromatographed into more volatile derivatives. The last aspect differs in principle from the others: whereas with the other procedures the volatility of the solute components and its amenability to GC is controlled by changing the properties and operating conditions of the chromatographic system, chemical derivatization alters the properties of the substance to be chromatographed.

When considering the problem of chemical derivatization in GC, it is expedient to distinguish between two causes that can affect the volatility of a substance. Low volatility can be due either to the fact that the substance has large molecules or that the molecules are mutually associated through polar groups. In the first instance, the intermolecular cohesion is the result of interactions by dispersion forces, and the volatility of such compounds obviously cannot be increased by derivatization. In the second instance, however, compounds with relatively small molecules can have very

low volatility if the molecule has functional groups that provide for polar interactions, especially interactions through hydrogen or ionic bonds. Many compounds of this type display a measurable volatility only at temperatures at which they decompose. Some of these compounds are considerably reactive and often decompose on contact with the active surface of a chromatographic support or with metals. These substances almost always give asymmetric chromatographic peaks. In such instances, marked enhancement of volatility and supression of the above undesirable effects can be achieved by effecting a derivatization that blocks the possibility of intermolecular association and reduces the reactivity of the compound. In addition, a conversion into a deliberately chosen derivative may impart to the molecule some properties that provide for selective separation or selective detection.

The combination of GC and chemical derivatization of the substances to be chromatographed is particularly useful in biochemical and biomedical investigations. Because of the wide applicability of GC and the various possibilities of coupling it with other analytical methods, particularly mass spectrometry, it is still of considerable importance although modern liquid chromatography is now used extensively.

This paper reviews the most important work on the preparation and applications of chemical derivatives in analytical GC. In the first part (Section 2), general aspects of the preparation and use of derivatives are dealt with and the most important and most frequently employed derivatives are discussed. In the second part (Section 3), the application of derivatives of individual groups of compounds are considered. The review does not cover reaction GC or pyrolysis GC.

2. GENERAL PART

2.1. Reasons for using chemical derivatives in gas chromatographic analysis

Derivatization is usually carried out in order to increase the volatility of substances with boiling points that are too high, to reduce the adsorption of solutes on the support and column surface and to improve the separation. Special derivatives often provide for the selective detection of certain species of compounds or the separation of chemically very similar compounds, such as optical isomers.

Substances with high molecular weights and several functional groups in the molecule are usually not amenable to GC. Polar functional groups reduce the volatility of the compounds, which results in excessively long retention times or non-elution of the compounds. The volatility can be enhanced by decreasing the polarity by blocking the polar groups, so that the derivatives can be chromatographed with reasonable retention times.

In practice, the reverse case may also occur. It is often necessary to analyze substances of relatively high volatility such as lower carboxylic acids in natural samples, and substantial losses of the components being determined could occur during the preliminary treatment of the sample (extraction, removal of the extractant, etc.). Therefore, the conversion of these compounds into less volatile derivatives is advantageous from the point of view of both the preliminary isolation and the GC determination proper.

Many substances cannot be analyzed by GC owing to their thermal instability. Such substances decompose in the sample inlet port and produce several peaks in the

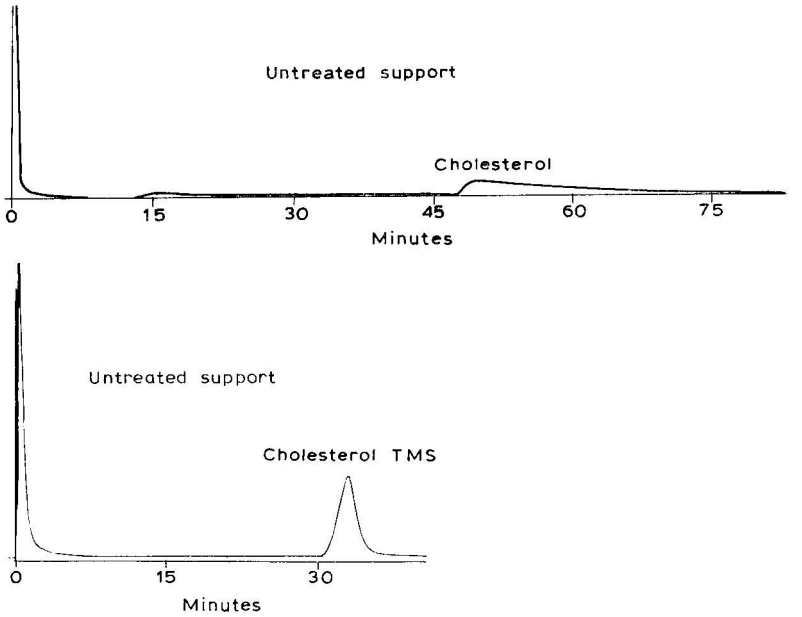


Fig. 1. Comparison of the chromatograms of free and trimethylsilylated cholesterol[557].

chromatogram. These difficulties can also be overcome by using suitable derivatives.

Compounds of high polarity and low volatility are usually very prone to adsorption on the chromatographic support or to decomposition when they come into contact with the latter. In these instances, the quantitative evaluation of the chromatogram is very difficult or even impossible. A classical example is the GC of cholesterol, which can be chromatographed as such or as the trimethylsilyl (TMS) derivative (Fig. 1). The broad and markedly tailing peak of free cholesterol could hardly be evaluated quantitatively, whereas the TMS ether gives a sharp symmetrical peak at an appreciably shorter retention time[557].

Tailing of peaks can also result when the concentration of the solute in the chromatographic system is too high. At high solute concentrations, the sorption isotherm is not linear and the peak is skewed. Even in these instances, the effect can be supressed by using suitable derivatives.

The adsorption of the solute on the support surface or column wall usually results in non-linearity of the calibration graph, especially when working at low solute concentrations in the sorbent and employing peak heights as quantitation parameters. Fig. 2 shows the dependence of the ratios of the peak height of morphine to that of squalene and the peak height of TMS-morphine to that of squalene on the size of the sample charge. With TMS-morphine this dependence is linear, but with free morphine it is non-linear owing to the above adsorption effects. Thus, the use of a suitable derivative can result in a linear dependences of the peak height on the sample charge even at very low solute concentrations[238].

Adsorption on the support and the tailing effect are often caused by carboxyl and hydroxy groups, especially with polyfunctional compounds of higher molecular weight. Amino and imino groups also interact strongly with the support. It is therefore

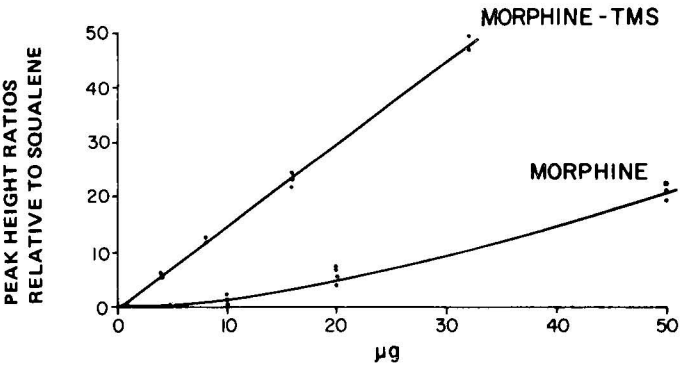


Fig. 2. Effect of silylation on the linearity of the plot of peak height *versus* amount of solute[238].

desirable to convert these groups into groups of lower polarity before the analysis proper. The carbonyl group usually does not interact strongly with the support and only rarely presents difficulties. It is derivatized either for special purposes or when its enol form is sufficiently stabilized with the neighbouring groups. Steric hindrance of the polar groups usually reduces their adverse effects, which sometimes may be completely absent so that derivatization is unnecessary. When employing the usual methods of derivatization, the above groups usually react with difficulty, and it is then necessary to employ special procedures.

Derivatization is of great importance in improving the separation of closely related compounds, and it frequently makes it possible to resolve compounds that cannot otherwise be separated. Sterols which differ in the position of the hydroxy group can serve an example. Isomers with a hydroxy group in the α-position are not separated from the β-isomer on non-polar columns, but when the hydroxyl group is

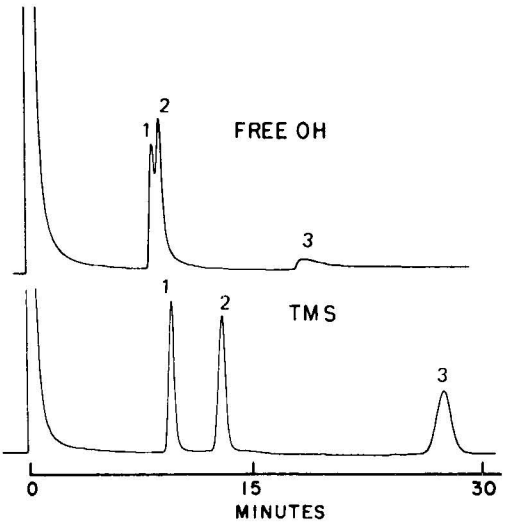


Fig. 3. Effect of silylation on the resolution and peak shapes of estrone (1), estradiol (2) and estriol (3) chromatographed on a non-polar stationary phase[557].

converted into a suitable derivative, both isomers are well resolved even on non-polar columns. The separation on a non-polar column of the three estrogens estrone, estradiol and estriol, as such and as their TMS derivatives, is shown in Fig. 3. While only incomplete separation with tailing peaks was achieved with the free compounds, the TMS derivatives were well resolved with symmetrical peaks[557].

Similarly, diverse derivatives can be employed in order to resolve different closely related compounds, such as positional or optical isomers, the separation of which usually presents difficulties. The types of derivative employed and examples are described with the individual groups of compounds in Section 3.

Special derivatives are used in order to increase the detector response. Chlorinated and fluorinated derivatives usually yield very high responses when detected with an electron-capture detector (ECD)[364] and these derivatives are often used in trace analysis. Compounds that contain phosphorus or nitrogen can be used with detectors sensitive to these elements[224].

2.2. General principles of the preparation of derivatives[238]

The preparation of derivatives before GC analysis is a potential source of errors that may affect the entire analytical procedure. For this reason, a thorough knowledge of the reactions used and the factors that influence their results is necessary. Special attention must be paid to the purity of solvents and reagents, the stability of the derivatives and reaction rates.

2.2.1. Technique of sampling

The method of sampling may be a serious source of errors in any analytical method and is critical in GC analysis. In addition to the problems of ensuring adequate homogeneity of the sample and taking a representative sample, there is a problem of its transfer into the gas chromatograph. It is necessary to ensure that the sample is not subject to decomposition or any reaction prior to the preparation of derivatives or the injection of a sample charge into the gas chromatograph. Items that are decisive for the success of the method are a knowledge of the kinetics of the reactions involved, removal of impurities from the solvents and reagents employed and, if necessary, the use of stabilizers. Although these aspects may seem obvious, it is because they are not strictly adhered to that many workers do not obtain satisfactory results.

2.2.2. Purity of solvents and reagents

Impurities that are introduced into the sample during cleaning, extraction, etc., appear as interfering peaks in the chromatogram and complicate the analysis or even make it impossible. If thin-layer chromatography (TLC) is employed for the preliminary clean-up of the sample, a blank experiment should be carried out by determining the impurities present in the adsorbent used; after the extraction of an amount of the adsorbent with the solvent, the condensed extract is derivatized and analyzed in the same manner as in the GC analysis proper. Interfering substances in the solvents and other chemicals used in the analysis are determined in a similar way. If some of the materials employed give peaks that overlap with those derived from the sample being analyzed, it is necessary to use chemicals of a higher grade of purity or, if necessary, to re-purify them.

2.2.3. Stability of derivatives

Some derivatives decompose by the action of heat, moisture, light, etc., when stored for a long period. In these instances, derivatization has to be carried out just before the analysis. This prerequisite is particularly important with derivatives that are very sensitive to moisture, such as trimethylsilyl derivatives.

2.2.4. Reaction rate

If quantitative analysis is concerned, the degree of conversion in the reactions involved should be known. Usually it is not known, and it is then necessary to assume that the reaction rates with the standard and the component being determined are identical. In this event, however, the conditions of reaction, such as the temperature, reaction time, type of catalyst and concentrations of the reactants, must also be the same in each instance. Sometimes the products of the decomposition of reagents may adversely affect the yield of a reaction and it is then necessary to store the chemicals in such a way that they can always be made available in a sufficiently fresh state.

2.3. Derivatives employed

2.3.1. Esters

Methyl esters are the most commonly used derivatives of the carboxylic group. The volatility of methyl esters is sufficiently high to permit the GC determination of even higher fatty acids[572]. However, with short-chain fatty acids, the volatility of the methyl esters is unsatisfactory as it can cause losses of the derivatives before analysis. Methyl esters are usually sufficiently stable, only the esters of some keto acids decomposing at about 100° in GC[172].

There are several methods for the preparation of methyl esters. Perhaps the most widely used are those which employ diazomethane[456,468] and a methanolic solution of boron trifluoride[394,410]. Also frequently used are reactions with hydrochloric acid and methanol[526] and sulphuric acid and methanol[359], by which means triglycerides are converted directly into the methyl esters of their fatty acids. Further, methyl esters are prepared by the pyrolysis of tetramethylammonium salts in the injection port[31,256], by methylation with 2,2-dimethoxypropane with the addition of dimethyl sulphoxide in order to inhibit the polymerization of the reagent[509], methylation on an ion exchanger[282] and other methods[106,157,585].

Vorbeck *et al.*[572] compared the yields obtained with different methylation methods (Table 1). With both lower and higher fatty acids, the best results were obtained by using the diazomethane method.

Diazomethane method. The reaction proceeds as follows:

$$R\text{–}COOH + CH_2{=}N{=}N \rightarrow R\text{–}COOCH_3 + N_2$$

Usually use is made of an ethereal solution of diazomethane prepared by the decomposition of N-nitroso-N-methyl-*p*-toluenesulphonamide with alkali. The ethereal solution of diazomethane is added gradually to the ethereal solution of the compounds to be derivatized until the reaction mixture remains permanently yellow. Another method consists in using three mutually connected bubble-through vessels which are swept with dry nitrogen. The first vessel contains diethyl ether, the second an

TABLE 1

COMPARISON OF YIELDS OBTAINED IN DIFFERENT METHODS OF METHYLATION

For butyric to caproic and for myristic to linoeic acids, the standard deviations were 0.32, 26.5 and 13.8, and 0.25, 0.77 and 0.52% with diazomethane, CH_3OH–HCl and CH_3OH–BF_3, respectively.

Acid	*Concentration given (wt.-%)*	*Concentration found (wt.-%)*		
		Diazomethane	*CH_3OH–HCl*	*CH_3OH–BF_3*
Butyric	38.7	38.6	4.0	30.1
Valeric	30.0	29.8	14.8	24.4
Caproic	31.1	31.6	29.6	30.4
Myristic	14.2	14.7	13.8	13.9
Palmitic	16.1	15.9	15.7	15.6
Stearic	17.8	17.6	18.7	18.0
Oleic	17.8	17.6	18.7	18.0
Linoleic	17.8	17.8	17.8	17.8

ethereal solution of 2-(2-ethoxyethoxy)ethanol plus an aqueous solution of potassium hydroxide, and the third an ethanolic solution of the acid. On adding N-nitroso-N-methyl-*p*-toluenesulphonamide to the second vessel, diazomethane is evolved and introduced into the third solution until a yellow coloration is attained. The excess of diazomethane is removed with a stream of pure nitrogen[487]. Fales *et al.*[175] used successfully the apparatus shown in Fig. 4. After injecting the reagents through the septum, the diazomethane produced within the inner tube dissolves in the diethyl ether or other liquor at the bottom of the other tube. The lower part of the apparatus is chilled with ice.

The diazomethane method is advantageous because of its simplicity and the

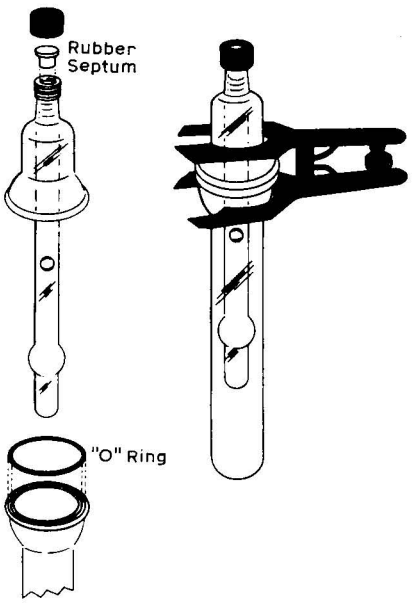


Fig. 4. Arrangement for methylation with diazomethane[175].

ease of preparation of methyl esters. As diazomethane reacts with water, it is necessary to work in water-free media. The formation of polymethylene polymers[572] can be prevented by employing vessels with a clean and smooth inner surface. A disadvantage of diazomethane is its toxicity and high reactivity, which often causes explosions. Therefore, ethereal solutions of diazomethane must be either used immediately or stored for only a short period at −20°.

Methanol method: catalysis with boron trifluoride. This method is represented by:

$$R\text{–}COOH + CH_3OH \xrightarrow{BF_3} R\text{–}COOCH_3 + H_2O$$

After adding the reagent to the sample of the acid, the reaction is completed by boiling the mixture for 2 min on a water-bath. On adding diethyl ether and water, the mixture distributes itself into two phases. After the removal of the aqueous layer, the ethereal phase is filtered, concentrated by heating on a water-bath and chromatographed. The reagents used are readily available and the mixture is sufficiently reactive even towards strongly hindered groups[394]. In some instances, boron trichloride[67] is more suitable than boron trifluoride.

Methanol method: catalysis with hydrochloric or sulphuric acid. The reaction is

$$R\text{–}COOH + CH_3OH \xrightarrow{HCl/H_2SO_4} R\text{–}COOCH_3 + H_2O$$

The preparation is carried out as in the preceding case. The methanolic solution of the sample is mixed with hydrochloric or sulphuric acid and is then refluxed for 2 h on a water-bath. The reaction mixture is again extracted with diethyl ether and the extract is cleaned, condensed and eventually injected into the gas chromatograph.

Pyrolysis of tetramethylammonium salts. This method is based on the reaction

$$R\text{–}COOH + (CH_3)_4N\text{–}OH \xrightarrow{360\text{–}400^\circ} R\text{–}COOCH_3 + H_2O + (CH_3)_3N$$

Usually, a sample of the acid is titrated with a methanolic solution of tetramethylammonium hydroxide on phenolphthalein. The solution is either injected directly into the GC inlet port warmed up to 360–400°[31], or placed in a 3-μl capillary, dried at 100° and eventually pyrolyzed at a higher temperature. Trimethylanilinium hydroxide in methanol can also be used as the reagent[69,536].

Higher esters are used for the GC of lower acids, where a higher volatility of the methyl esters can cause losses during preparation and erroneous quantitative results. Butyl[215] and benzyl[332] esters are very frequently used, but other esters have also been studied: ethyl and propyl esters[22], substituted benzyl esters[556,578], esters that give a high response with the electron-capture detector, such as β-chloroethyl[312], pentafluorobenzyl[319], and hexafluoroisopropyl esters[160], and others. L-Menthyl esters[1] were used to distinguish optical antipodes.

The methods of the preparation of higher esters are similar to those for methyl esters. A commonly used method is the esterification of the carboxylic group by reaction with an alcohol, catalyzed by boron trifluoride or hydrochloric or sul-

phuric acid. Felder *et al.*[178] esterified a number of acids with methanol and higher alcohols. The reaction was catalyzed by pyridine with a large excess of N,N′-dicyclohexylcarbodiimide, which acted as a dehydration agent:

$$RN{=}C{=}NR \xrightarrow{H^+} RN{=}C{=}\overset{+}{N}HR \xrightarrow{R_1COO^-} RN{=}C(O{-}CO{-}R_1){-}NHR \xrightarrow{R_2OH} R_1{-}COOR_2 + RNH{-}CO{-}NHR$$

If a precipitate of dicyclohexylurea is produced in the reaction, it is allowed to settle and the clear solution is injected into the gas chromatograph. Felder *et al.*[178] obtained quantitative results with a number of fatty, pyrazine and halobenzoic acids. The use of higher diazoalkanes[118,587] or diazotoluene[117] has also been described. Their ethereal solutions are not as explosive as those of diazomethane and they can be stored at low temperatures for longer periods. Good results were achieved by Thenot *et al.*[548], who esterified carboxylic acids by reaction with N,N′-dimethylformamide acetals:

$$R{-}COOH + \begin{matrix} R'{-}O \\ \diagdown \\ CH{-}N(CH_3)_2 \\ \diagup \\ R'{-}O \end{matrix} \xrightarrow{-R'{-}OH} R{-}COO^- + R'{-}O{-}\overset{+}{C}H{-}N(CH_3)_2 \rightarrow$$

$$\rightarrow RCOOR' + HCON(CH_3)_2$$

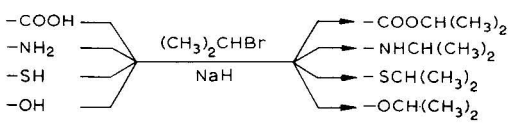
The reaction is sufficiently quantitative for analytical purposes and the alkyl group R′ can be varied widely.

Other methods of preparation of esters for GC include the alcoholysis of the imidazolides of acids, which are prepared by the reaction of the acid with N,N′-carbonyldiimidazole[335]:

and the reaction of alkyl iodides with the tetramethylammonium salts of acids[223]:

$$R{-}COOH \xrightarrow{(CH_3)_4N^+OH^-} R{-}COO^-(CH_3)_4N^+ \xrightarrow{R'{-}I} R{-}COOR'$$

Special mention can be made of isopropyl esters, which are prepared by reaction with 2-bromopropane and sodium hydride. These derivatives were introduced by Pettitt and Stouffer[448]. It is interesting that this mixture of reagents can also derivatize other functional groups in the molecule, which can be of practical importance in the analysis of compounds with different functional groups:



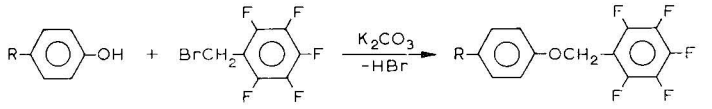
Up to now this reaction has been little utilized for derivatization in GC analysis, and it may possibly find wider application.

2.3.2. Ethers

This type of derivatization is used to protect hydroxy groups. Apart from TMS ethers, which will be dealt with separately, ethers have not been widely used as derivatives in GC analysis and are employed for special purposes. Methyl ethers o) saccharides are prepared by reaction with methyl iodide in the presence of silver(If oxide in dimethylformamide[325]. An ethereal solution of potassium *tert.*-butanolate has been used[110] instead of silver(I) oxide.

$$2\ R\text{–}OH + 2\ CH_3I + Ag_2O \xrightarrow{\text{catalyst}} 2\ R\text{–}OCH_3 + 2\ AgI + H_2O$$

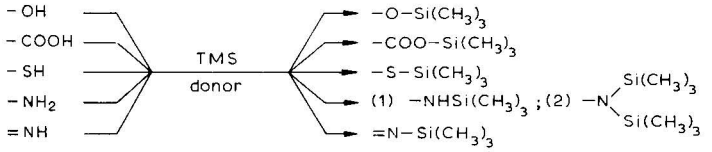
Earlier, the method was often used mainly to derivatize high-molecular-weight hydroxy and polyhydroxy compounds. Derivatives for the trace analysis of hydroxy compounds are prepared in a similar way; the reaction of phenolic compounds with α-bromo-2,3,4,5,6-pentafluorotoluene results in an ether that gives a high ECD response. This reaction is catalyzed by potassium carbonate[318]:



Similarly, reaction with 1-fluoro-2,4-dinitrobenzene gives 2,4-dinitrophenyl ethers[112].

2.3.3. Silyl derivatives

These derivatives have been most widely used in the GC of non-volatile substances. In particular, the trimethylsilyl (TMS) group can be used in order to block diverse polar groups. The preparation of the derivatives is represented by the scheme



If the enolized carbonyl group is included with the above range of functional groups, virtually all groups that may complicate the GC analysis owing to their polarity are covered. The advantage of trimethylsilylation is evident particularly with compounds that have different functional groups in the molecule, as all of the groups can be derivatized by a one-step reaction.

The methods of preparation of TMS derivatives have been described in detail. In his monograph, Pierce[449] presented a number of different modifications of the preparations of TMS derivatives according to the reagents and functional groups. The following reagents were used:

(1) trimethylchlorosilane (TMCS), alone or with an acceptor of the acid or, if necessary, with a catalyst;

(2) hexamethyldisilazane (HMDS), mostly with TMCS or another catalyst;

(3) silylamines, such as trimethylsilyldiethylamine (TMSDEA) and trimethylsilylimidazole (TMSIM);

(4) silylamides and other reagents; most commonly employed are N,O-bistrimethylsilylacetamide (BSA), N,O-bistrimethylsilyltrifluoroacetamide (BSTFA) and hexamethyldisiloxane (HMDSO).

The frequently used mixture of HMDS and TMCS in pyridine[532] is a relatively weak TMS donor; stronger reagents, mainly BSA, BSTFA[523] and TMSIM, are employed in order to derivatize strongly hindered groups and groups of low reactivity. Other analogous reagents, N-methyl-N-TMS-trifluoroacetamide and N,N,N′,N′-tetrakis-TMS-1,*n*-diaminoalkanes, were introduced by Donike[144,145].

Pyridine is often used as reaction medium, but it is difficult to chromatograph owing to the tailing of peaks and its peak can overlap with some lower derivatives. Other solvents used include acetonitrile and dimethylformamide. During derivatization, it is necessary to maintain strictly anhydrous conditions as even trace amounts of water decompose TMS derivatives into the parent compounds. Weiss and Tambawala[580] described a method of silylation in the presence of water, but the principle consists in adding such a large excess of the reagent that the amount of water present becomes negligible.

The reaction is usually performed in flasks sealed with silicone rubber closures, and the introduction of the reagent and withdrawal of samples are carried out through the stepum with the use of an injection syringe. Because of the sensitivity of the derivatives towards water, it is necessary to prepare them immediately before analysis, although the derivatives have been reported to remain stable for several days under anhydrous conditions[276]. A method has also been described for the preparation of TMS derivatives on the column[168,387]. The sample charge is followed by a charge of the silylation agent, the conditions being chosen such that the components to be separated are freed from water and alcohol when entering into the reaction. The TMS derivatives produced in this way then migrate down the column and are separated. An apparatus for the removal of pyridine from the sample was described by Lehrfeld[355].

The choice of the stationary phase for the separation of TMS derivatives is usually not critical. Use has been made of diverse stationary phases, but non-polar and non-selective phases are to be preferred[449]. The support is usually deactivated, by washing it with an acid followed by silanization, which helps to produce a higher separation efficiency of the column[397]. The packing must not be acidic in case the derivatives decompose. Some less stable derivatives can decompose upon contact with metallic parts of the instrument and it is therefore recommended that all-glass apparatus be employed; the column, however, may be made of glass or stainless steel[205]. When using a flame-ionization detector, an aerosol of silicon dioxide is produced on combustion of siliceous substances and is deposited on the electrodes; this deposit can decrease the sensitivity of detection or alter the response factors. When using BSTFA, this effect is reduced owing to the formation of volatile silicon tetrafluoride.

Halomethyldimethylsilyl derivatives and dimethylsilyl derivatives[530,531] are prepared in a similar way. They have shorter retention times than TMS derivatives, but are less stable[238]. Some examples are shown in Table 2.

TABLE 2

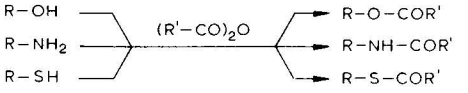
COMPARISON OF RETENTION TIMES OF SOME HIGHER ALCOHOLS, PHENOLS AND THEIR DMS AND TMS DERIVATIVES

Conditions: 6 ft. × 4 mm I.D. glass column; 15% Apiezon L on Gas-Chrom P, 100–120 mesh; 120°; nitrogen carrier gas, flow-rate 60 ml/min. Retention times are given relative to hexadecanol and its derivatives and phenol derivatives, respectively. Values in parentheses are absolute retention times.

Compound studied	*Relative retention time*		
	Parent compound	*DMS derivative*	*TMS derivative*
Dodecanol	0.17	0.19	0.19
Tetradecanol	0.42	0.43	0.44
Hexadecanol	1.00 (14.0 min)	1.00 (8.9 min)	1.00 (10.4 min)
Octadecanol	–	2.29	2.28
Phenol	–	1.00 (8.5 min)	1.00 (11.2 min)
o-Cresol	–	1.73	1.74
m-Cresol	–	1.85	1.78
p-Cresol	–	1.98	1.98

2.3.4. Acyl derivatives

Acyl derivatives are common derivatives of hydroxy, amino and thiol groups:



Halogenated acyl derivatives have found the widest application owing to their high ECD response and utility in trace analysis[447].

Acyl derivatives are prepared by reaction with an excess of the acylation reagent (usually the anhydride of the corresponding acid) in pyridine, tetrahydrofuran or another solvent that is able to bind the acid produced. The type and amount of solvent used frequently have a significant influence on the yield of the reaction[459]. The reaction mixture is then usually heated for 1/2–1 h at 60° in order to evaporate the solvent, and the concentrate is injected into the gas chromatograph. It is essential to work under anhydrous conditions as the derivatives are hydrolyzed on contact with water. Trace amounts of water are removed by the excess of solvent, which protects the derivatives against hydrolysis. The chloride of an acid can also be used as an acylation agent. Advantageous acylation agents are acylimidazoles[279]; by-product imidazole is relatively inert and does not decompose the derivatives. Anders and Mannering[15] prepared acetyl and propionyl derivatives directly in the column by injecting the sample and anhydride consecutively. Different retention times of the derivatives were utilized in order to identify some alkaloids and steroids.

Acetyl derivatives. Acetyl derivatives were mainly used in earlier work. Their significance lies in the ready availability of the reagents. Nowadays, halogenated acetyl derivatives are more common owing to their high affinity for electrons and the possibility of carrying out high-sensitivity analyses with the use of ECDs. Landowne and Lipsky[346] arranged the haloacetyl esters of sterols in order of increasing ECD response: trifluoroacetate < trichloroacetate < bromoacetate < dichloroacetate <

chloroacetate. However, chloroacetyl derivatives have some unfavourable properties, such as the formation of asymmetric peaks[108], so that trifluoroacetyl (TFA) derivatives are used more frequently. In addition to the above methods, TFA derivatives can be prepared by a method described by Donike[146]: trifluoroacetylation is carried out with N-methyl-bis-TFA-amide, which reacts with $-NH_2$, -OH and -SH groups under mild conditions. As the reagent is a liquid, no solvent is necessary. The excess of the reagent protects the derivatives against hydrolysis.

Halogenated acyl derivatives of higher acids usually afford as a rule even more sensitive analyses than do TFA derivatives. McCallum and Armstrong[384] compared the responses of seven derivatives of thymol: 2,4-dinitrophenyl and pentafluorobenzyl ethers and heptafluorobutyryl, pentafluoropropionyl, chloroacetyl, fluoroacetyl and pentafluorobenzoyl esters. The most sensitive analysis was achieved with pentafluorobenzoate, with which it was possible to determine as little as 1 pg of thymol. The above situation is shown in Table 3. Heptafluorobutyrates (HFB) give a lower sensitivity, but they have very high volatility and are used in practice mainly with natural amines and alcohols of low volatility[595].

TABLE 3

RELATIVE SENSITIVITY OF THE ELECTRON-CAPTURE DETECTOR TOWARDS DIFFERENT DERIVATIVES OF THYMOL[384]

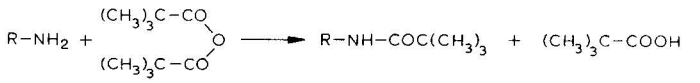
Conditions: 1 m × 2 mm I.D. glass column; 1% SE-52 on Diatoport S; nitrogen carrier gas, flow-rate 15–20 ml/min.

Derivative	*Column temperature (°C)*	*Retention time (min)*	*Relative sensitivity**
Heptafluorobutyrate	70	2.7	1.0
Pentafluoropropionate	70	1.9	1.3
Monochloroacetate	100	3.1	0.3
Monofluoroacetate	100	1.2	$7 \cdot 10^{-3}$
Pentafluorobenzyl ether	100	5.8	5.9
Pentafluorobenzoate	150	1.7	6.9
2,4-Dinitrophenyl ether	150	9.8	0.3
Free thymol detected with FID	120	3.0	$7 \cdot 10^{-4}$**

* Relative to heptafluorobutyrate.

** Relative to the ECD response of thymol heptafluorobutyrate.

N,O-Dipivalyl esters are prepared by a procedure similar to that described above, and have been used for the analysis of thyroid hormones[288]. Both amino and hydroxy groups are converted into the pivalyl derivative:



The derivatives are stable and can be cleaned up prior to GC, *e.g.*, by TLC.

2.3.5. Oximes and hydrazones

The carbonyl group usually does not present any special difficulties in GC

analysis. However, the peaks of carbonyl compounds often overlap with those of interfering components[570], or the presence of the carbonyl group can sometimes be the cause of instability of compounds and bring about asymmetric peaks. In these instances, the carbonyl group must be converted into an inert derivative. Oximes proved to serve this purpose very well:

They are usually prepared by reaction of the reagent (hydroxylamine, methoxylamine or benzyloxyamine hydrochloride) with the carbonyl compound in pyridine. The reaction is allowed to proceed either at ambient temperature overnight or is accelerated by warming the mixture to 60–100°. The pyridine is removed with a stream of nitrogen and the sample is dissolved in another solvent (ethyl acetate) prior to its introduction into the gas chromatograph; if necessary, other groups can be blocked. Oximes alone (R = H) are used only rarely. Lohr and Warren[363] noticed that different oximes decomposed to the corresponding nitriles on the column, the decomposition being dependent on temperature. At 250°, the decomposition is complete and the chromatograms of the products are reproducible. However, methoximes have found much wider application[546], particularly for protecting labile keto groups in substances with higher molecular weights (steroids). Compared with the parent compounds, methoximes are more stable, do not decompose during analysis and can be further modified chemically according to the type of interfering group. Higher oximes, such as O-butyloximes, O-pentyloximes and O-benzyloximes, have also been studied and are important when combining GC with mass spectrometry. They are sufficiently stable and their characteristic mass spectra can be easily interpreted quantitatively[32].

Good chromatographic properties have been encountered with hydrazones of carbonyl compounds:

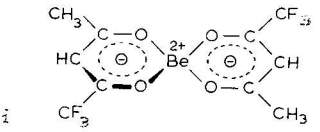
They are prepared by the reaction of a substituted hydrazone with the sample in the presence of a catalyst, usually acetic acid. At ambient temperature, the conversion is complete in 1–2 h. The excess of the reagent is usually removed with a stream of nitrogen and the derivative, dissolved in a suitable solvent, is injected into the gas chromatograph. The derivatives can also be prepared by use of Girard T reagent[199]. The direct GC analysis of hydrazones is carried out only with special derivatives. For instance, 2,4-dinitrophenylhydrazones and 2,4,6-trichlorophenylhydrazones have a sufficiently high ECD response and can be used in trace analysis[297]. In addition, hydrazones are used for the preliminary isolation of carbonyl compounds; in GC analysis, they are injected together with α-ketoglutaric acid or another keto compound that liberates the carbonyl compound, the latter being chromatographed in its free state.

2.3.6. Chelates of metals

Ions of metals can be analyzed by GC in the form of their volatile compounds. Chlorides[528,604] and fluorides[306] have sufficient volatility for this purpose, but special

reactors have to be installed ahead of the column. Jones and Nickless employed arylmercury[301] and methylmercury[302] compounds for the determination of mercury, and Tatton and Wagstaffe[538] analyzed dithizonates of mercury. Segard *et al.*[494] described the GC separation of 13 arenetricarbonic complexes of chromium. However, these compounds are interesting from the point of view of the chemistry of complexes rather than for their analytical utility. Schwedt and Ruessel[491] converted arsenic in biological tissues into triphenylarsine and analyzed it by GC.

Chelates of metals have found much wider application. They are prepared by using β-diketones of the acetylacetone type[507], but other similar compounds, such as β-ketoamines[269] and monothioacetylacetone[38], can also be employed. The active hydrogen of the methylene group of β-diketones is substituted by the metal, and the second co-ordination bond is formed by the oxygen of the ulterior keto group. This gives rise to a stable six-membered heterocycle; an example of a beryllium complex shows the saturation of four co-ordination bonds by two molecules of β-diketone:



Chelates are prepared by reaction of the diketone with the sample, the mixture being extracted (usually with benzene) and the extract chromatographed. By use of fluorinated diketones and an ECD, it is possible to attain a high sensitivity of analysis, but the linearity of the response is poor[5,477].

The earliest used and most studied chelate-producing reagent is acetylacetone and its trifluoro and hexafluoro derivatives. These reagents have been used for the preparation and determination of complexes of beryllium[329,475,507], aluminium[407,493,507], chromium[214,476,478], copper and iron[329,493], rare-earth metals[507] and other metals[407]. Several workers have demonstrated the application of this approach to the determination of toxic beryllium in diverse biological materials[541,592], blood[540] and urine[186]. Eisentraut *et al.*[165] determined beryllium in lunar and meteoric samples by this means. The high-sensitivity determination of chromium in biological samples[62,245,474,485] has also been described.

Higher volatility is displayed by chelates with pivaloyltrifluoroacetylacetone (2,2-dimethyl-6,6,6-trifluorohexanedione-3,5), used for the determination of rare-earth metals[501,537] and other trivalent metals[41]. 2,2,6,6-Tetramethylheptadione-3,5 (THD) has been employed with rare-earth metals[490]. In the analysis of calcium and strontium, the situation is complicated by the formation of mixed chelates (dimers):

$$[Ca(THD)_2]_2 + [Sr(THD)_2]_2 \leftrightharpoons 2\ CaSr(THD)_4$$

Highly fluorinated chelate-producing agents are employed in trace analysis with the use of an ECD. 1,1,1,2,2,3,3-Heptafluoro-7,7-dimethyloctadione-4,6 forms, with rare-earth metals, iron, nickel, chromium, copper and other metals, complexes that have excellent chromatographic properties, high volatility and stability and a high ECD response, enabling the ultramicro analysis of these elements to be performed[185,506,521]. Highly fluorinated diketones, such as decafluoroheptanedione-3,5 and dodecafluorooctanedione-4,6, were employed in the preparation and high-sensitivity analysis of

Corrected V.P. / 6. July 1976

mixed complexes of rare earths; tri-*n*-butyl phosphate and di-*n*-butyl sulphoxide were used as partner ligands. In addition to lanthanides[34,84,88], uranyl and thorium[505] have been determined in this way.

Of other reagents, use has been made of hexafluoromonothioacetylacetone for the preparation of volatile derivatives of platinum metals[38] and nickel [35,44] in particular, and of bisacetylacetone methylenediimine for nickel, palladium and platinum[43]. Further references and information can be found in a monograph by Moshier and Sievers[412] published in 1965, and in more recent reviews[42,123].

3. SYSTEMATIC PART

3.1. Alcohols and phenols

Although the GC analysis of lower alcohols is no longer a major problem, the presence of the hydroxy group results in considerable adsorption on some kinds of support, which leads to peak tailing and renders quantitative analysis impossible, especially with aromatic hydroxy compounds. In addition, in trace analysis it is necessary to prepare derivatives with a high detector response and, in the analysis of optical antipodes, derivatives that will permit their resolution. Zarazir *et al.*[599] presented the retention data of various derivatives of a number of alcohols on three stationary phases. On plotting these data on a triangular diagram, it was possible to identify unknown compounds.

3.1.1. Esters

Studies have been made of the acetates of alcohols[232], glycols and polyethylene glycols[233] and some phenolic compounds[217] as the most accessible derivatives. Decroix *et al.*[136] used benzoyl esters for the determination of glycerol, as it is not necessary to use an anhydrous medium. However, the derivatives decompose on the column and special conditions must be observed during the analysis.

Halogenated acyl derivatives are frequently employed in order to achieve selective detection. Argauer[23] described the GC of 32 phenols as their chloroacetyl esters, and Larkham and Pagington[351] used these derivatives to prove the presence of trace amounts of alcohols in tobacco smoke. TFA esters of phenols have also been studied, but they are rather unstable[503]. Trace amounts of water and acids cause their decomposition, and sterically hindered groups do not react quantitatively. These derivatives are of advantage with compounds that have different functional groups, for instance in the separation of ethanolamines[81] and metabolites of catecholamines[17]. Walle and Ehrsson[574] used HFB esters for the determination of picogram amounts of various alcoholic compounds.

Interesting derivatives have been described by Bassette *et al.*[37]. In order to increase the sensitivity of the determination of primary and secondary alcohols, they employed 2,6-dinitrophenylhydrazones of pyruvic acid. Neurath and Lüttich[423] studied the GC separation of esters of 4′-nitroazobenzene-4-carboxylic acid and gave the corresponding data for a number of alcoholic compounds.

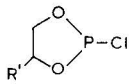
3.1.2. Ethers

Although phenolic hydroxy compounds were separated as the methyl esters in

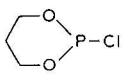
early work[93,94], these derivatives are used only in special cases today (except for TMS ethers), particularly for increasing the sensitivity of the determination. Haken and Khemangkorn[233] studied the GC behaviour of different ethers and ether acetates of propylene and ethylene glycols. Kawahara[318] investigated pentafluorotolyl ethers of several alcohols and phenols, which derivatives he prepared by using α-bromo-2,3,4,5,6-pentafluorotoluene, and showed that they had a number of excellent chromatographic properties. These ethers are stable in water, give an excellent ECD response, which is specific in the presence of impurities, and they are particularly suitable for trace analysis. Other workers have studied 2,4-dinitrophenyl ethers[112] and 2,6-dinitro-4-fluoromethylphenyl ethers[495] of phenols. The quantitativeness of their preparation is worse than that of the above derivatives, the yields varying in the range 50–70%, depending on the type of phenol involved.

3.1.3. Heterocyclic derivatives

Vilceanu and Schulz[567] developed phosphorus-containing heterocyclic derivatives for the high-sensitivity detection of lower alcohols with an alkali flame-ionization detector. The derivatives (III, IV) were prepared by reaction with 2-chloro-1,3,2-dioxaphospholane (I) and 2-chloro-1,3,2-dioxaphosphorinane (II):



(I)



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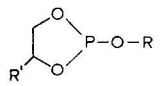
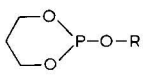


(III)



(IV)

These derivatives are especially suitable for the determination of alcohols in anhydrous and alcohol-free media.

3.1.4. Trimethylsilyl derivatives

These derivatives have been studied in detail in the GC of alcohols, owing to their chromatographic properties and easy preparation. As the reactivity of the hydroxyl group is sufficiently high, except for some special instances, it is generally possible to employ any silylation agent. For the derivatization of the less reactive tertiary alcohols, the formerly used HMDS[348,349] has been replaced by a more reactive mixture of HMDS and TMCS[191]. The preparation of volatile derivatives of glycols[356] and polyethylene glycols[24,169,591] is carried out mainly with BSA or, if necessary, BSTFA. The use of these derivatives in the determination of glycerol in lipids[458], higher terpenic alcohols[496,579] and fatty alcohols[593,601] has been studied. With some compounds, *e.g.*, prostaglandins, use was made, in addition to TMS ethers alone[305], of a combination of them with methyl or acetyl derivatives[6] or with cyclic *n*-butyl boronates[433]. The TMS derivatives of phenolic hydroxy compounds are easy to prepare, can be well separated and have other good chromatographic properties[190,350,511]. Their application is very important with polyhydroxy compounds of natural origin, such as hydroxy-[542] and polyhydroxyanthraquinones[197], gossypol[385], morphine[15,70,373,588], flavonoids[195,315], aminochromes[257] and others[516]. The use of trimethylsilylation with many other hydroxyl compounds has been quoted in Pierce's monograph[449].

3.1.5. Derivatives of optically active substances

Pereira *et al.*[442] employed *R*-(+)-1-phenylethyl isocyanate for the preparation of carbamates of optically active C_4–C_{10} alcohols. The individual derivatives of diastereoisomers have sufficiently different retention times to be resolved by GC. Other esters have also been used for this purpose. Anders and Cooper[14] described esters of 3β-acetoxy-Δ^5-etienic acid, and Hammarström and Hamberg[239] studied D-phenylpropionates of higher alcohols. Brooks *et al.*[73] employed sesquiterpenic drimanoyl chloride and monoterpenic chrysanthemoyl chloride for the preparation and resolution of esters of enantiomers of alcohols.

3.2. Aldehydes and ketones

In a similar manner to blocking amino groups by condensation with a carbonyl group, aldehydes and ketones can be modified for GC analysis by reaction with a suitable amino compound. The carbonyl group can also be oxidized when determining the resultant ester, or converted into an acetal or ketal.

3.2.1. Enamines

The preparation of some enamines of di-*n*-hexyl and di-*n*-heptyl ketones and their chromatographic properties have been described by VandenHeuvel *et al.*[562]. They prepared, in a medium of ethyl acetate and with catalysis by acetic acid, condensates with N-aminopiperidine, N-aminohomopiperidine, pentafluorophenylhydrazine and phenylhydrazine.

2,4-Dinitrophenylhydrazones (DNPHs) of carbonyl compounds have frequently been applied in isolations prior to GC analysis. Ralls[460,461] prepared the 2,4-DNPHs of mixtures of aldehydes and ketones and, after warming the condensates with α-ketoglutaric acid, the carbonyl compounds released were separated by GC. This method was developed further by other workers[150,236,377]. Jones and Monroe[300] employed dimethylaminobenzaldehyde to liberate the carbonyl compound from 2,4-DNPH. Halvarson[235] described an apparatus for the regeneration of carbonyl compounds and their determination on a microanalytical scale. Gadbois and coworkers[198,199] prepared 2,4-DNPH derivatives by reaction with Girard T reagent and released the carbonyl compounds by using an excess of formaldehyde produced from paraformaldehyde or methylolphthalimide. Employing the above method, Ronkainen and Brummer[470] analyzed α-hydroxyketones. Their 2,4-DNPHs were pre-purified by steam stripping in the presence of concentrated sulphuric acid. The diketones produced on reaction with oxoglutaric acid were injected into the gas chromatograph.

The direct GC analysis of the 2,4-DNPHs of aldehydes and ketones has also been described[177,310,520]. These derivatives display good chromatographic properties and permit a high sensitivity of analysis to be attained. Johnson and Hammond[297] used 2,4,6-trichlorophenylhydrazine as a reagent. Employing these derivatives with the ECD, it was possible to detect 10^{-7}–10^{-10} g of carbonyl compounds. The direct separation of 27 carbonyl compounds by programmed-temperature GC has been described by Jack and Riess[289].

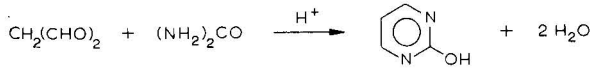
3.2.2. Oximes

Oximes are used almost entirely in special cases. Vogh[570] used them in the analysis of exhaust gases and utilized their acidic properties for their preliminary

isolation; hydrocarbons and other interfering components were extracted into pentane. However, oximes decompose to the corresponding nitriles[363] during GC, especially on contact with metals or their oxides. The separation must therefore be carried out in glass apparatus.

3.2.3. Other derivatives

Hamberg *et al.*[237] isolated and identified malonaldehyde after its condensation with urea, and converted the 2-hydroxypyrimidine produced, which has a low volatility but is stable, into a TMS ether. The reaction is



This method has been applied to the analysis of biological samples.

Schogt *et al.*[489] oxidized higher aliphatic aldehydes with silver oxide to the corresponding acids, which were subsequently separated after their esterification with diazomethane. Gray[221] synthesized dimethylacetals by refluxing aldehydes with a methanolic solution of hydrochloric acid and attained yields of over 95%. Acetals were oxidized to acids with chromium trioxide in glacial acetic acid and the acids were methylated with methanolic hydrochloric acid[222].

3.3. Amines

The GC of free amines, without special modifications to the column, is unsatisfactory owing to the spurious adsorption of the solute and the resulting peak tailing. Most commonly, the amino group is blocked by acylation, trimethylsilylation and the preparation of various condensation products (Schiff's bases).

3.3.1. Acyl derivatives

Anders and Mannering[15] described the chromatographic behaviour of acetyl and propionyl derivatives of substances that contain amino and hydroxy groups. They prepared the derivatives directly on the column by injecting the anhydride. In this way, it is possible to characterize some organic bases satisfactorily, but it is not possible to use this method for quantitative analysis. Nevertheless, Marmion *et al.*[372] described the application of this method to the determination of trace amounts of 2-naphthylamine in 1-naphthylamine by using a method of comparison with standards. Halogenated acyl derivatives have shown a much greater significance. A comparison of their properties and ECD responses was made by Clarke *et al.*[108]. It follows from their work that each species of derivative can be employed with certain species of amino compound; the highest sensitivity of analysis was achieved with HFB derivatives (Table 4).

Owing to their ease of preparation, trifluoroacetyl (TFA) derivatives of amines have great significance and have been used in the GC of aliphatic[124,411], alicyclic[360] and aromatic[104,151] amines and diamines[82,365,405]. This method has also been employed in the determination of biological amines in natural materials[50,109,316,422]. In a similar way, mono- and disubstituted ureas were determined[173]. The determination of compounds that contain several different functional groups is performed mostly through combination with other derivatives. Thus, Mori *et al.*[403] determined the

TABLE 4

COMPARISON OF THE CHROMATOGRAPHIC PROPERTIES OF SOME ACYL DERIVATIVES OF AMINES

Conditions, A: 6 ft. × 4 mm I.D. glass column; 6% QF-1 on Anakrom ABS, 60–70 mesh; column temperature 152°; carrier gas (nitrogen) flow-rate, 30 ml/min. Conditions, B: column as in A; temperature 155°; carrier gas flow-rate 80 ml/min.

Amine	*Derivative*	*Conditions*	*Retention time (min)*	*Peak shape*	*Sensitivity of determination**
Benzylamine	Acetyl	B	2.5	Asymm.	0.04
	Monochloroacetyl	B	3.0	Slightly asymm.	30
	Trifluoroacetyl	A	1.6	Symm.	0.8
	Pentafluoropropionyl	A	2.2	Symm.	229
	Heptafluorobutyryl	A	2.3	Symm.	715
α-Methylbenzylamine	Acetyl	B	2.5	Asymm.	0.16
	Monochloroacetyl	B	2.8	Slightly asymm.	32
	Trifluoroacetyl	A	2.2	Symm.	0.5
	Pentafluoropropionyl	A	2.2	Symm.	0.5
	Heptafluorobutyryl	A	2.2	Symm.	563

* Expressed as peak height (mm) per 10^{-9} mole of the compound.

products of the hydrolysis of copolyamides (diamines, diacids, amino acids) after their prior esterification with methanol–hydrochloric acid and acetylation with trifluoroacetic anhydride. Noguchi *et al.*[426] determined the metabolites of tryptophan in the form of their methoxy-TFA derivatives. Pentafluoropropionyl[17,314] and pentafluorobenzyl[401] derivatives were employed in the determination of trace amounts of amines in biological samples. Ephedrines[126], melathonines and indoleamines[138] and other biological amines[77] have been analyzed as their HFB derivatives.

3.3.2. Trimethylsilyl derivatives

The amine group is not very reactive in silylation reactions and is relatively difficult to silanize. In a mixture of hexuronic acid, 1-octanol and 1-octylamine, the amine gives the lowest yields[379] under different silylation conditions. However, the differences in reactivity are not significant and a number of amines can be chromatographed in the form of their silyl derivatives[449].

The above derivatives are prepared by the use of various silylation agents, mostly the more powerful agents. HMDS alone is usually inadequate for derivatizing secondary amine groups and is used in admixture with catalysts[181]. BSA is often employed[393], also with the addition of a catalyst. Butts[87] employed a mixture of BSTFA and 1% of TMCS for the preparation of the derivatives of amines and other compounds and tabulated their retention data. Maruyama and Takemori[375] prepared TMS derivatives of dopamines and related compounds by reaction with trimethylsilylimidazole in acetonitrile.

A more complicated situation is encountered with compounds that contain several amino groups of different types. Holmstedt *et al.*[270] studied the preparation of the TMS derivatives of tryptamine and related compounds. On applying BSA in

pyridine, mainly monosubstituted derivatives are produced, while the use of BSA–TMCS mixtures yields disubstituted derivatives as the major products. When a longer reaction time is allowed, however, comparable amounts of di- and trisubstituted derivatives are produced[559]. Albro and Fishbein[7] silylated metabolites of thyrosine and tryptophan with different agents. From the viewpoint of quantitativeness of the reaction and uniformity of the products, the most suitable agents are BSTFA and TMCS in pyridine, with the addition of TMSDEA if necessary.

In the molecules of catecholamines one or several hydroxy groups are present together with a primary or secondary amine group. Without blocking these polar groups, catecholamines cannot be analyzed by GC. Horning *et al.*[281] found that BSA and BSA–TMCS silylate the primary amine group to the second degree while the secondary amine group is converted slowly or not at all. In order to obtain uniform products, they employed TMSIM in acetonitrile, which silylates hydroxy groups. Upon subsequent addition of BSA–TMCS, primary amine groups are converted into bis-TMS derivatives while secondary amine groups remain unchanged. The amine group can also be blocked after silylation by TMSIM in another way. Horning *et al.*[279] used N-acylation with N-acetyl- and N-heptafluorobutyrylimidazole. Good results were obtained by blocking the amine group by condensation with a carbonyl compound[320]. Fig. 5 shows the chromatogram of a mixture of catecholamines. After trimethylsilylation of the hydroxy groups, the amine groups were converted into enamines by reaction with acetone. The secondary groups were not blocked[91].

3.3.3. Dinitrophenyl derivatives

These derivatives are used for the detection of trace amounts of amines with an ECD. Employing these derivatives, Weston and Wheals[582] determined about 1 ppm of cyclohexylamine in beverages and other materials. A more detailed study on the preparation and behaviour of these derivatives in GC has been described by Walle[573]. They are prepared by reaction with 1-fluoro-2,4-dinitrobenzene in benzene. Minimum amounts that can be determined lie in the range 2–20 pg. Good results were obtained by Edwards and Blau[163], who used 2,4-dinitrobenzenesulphonic acid for the preparation of these derivatives. Hydroxy groups present in various amines were blocked by silylation with BSA.

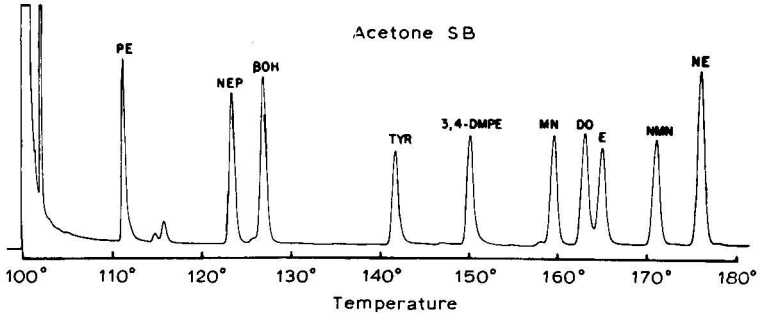


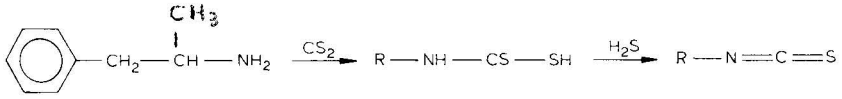
Fig. 5. Chromatogram of a mixture of catecholamines after their trimethylsilylation and conversion into enamines[91]. PE = β-Phenylethylamine; NEP = norephedrine; βOH = β-hydroxy-β-phenylethylamine; TYR = tyramine; 3,4-DMPE = β-(3,4-dimethoxyphenyl)ethylamine; MN = metanephrine; DO = dopamine; E = epinephrine; NMN = normetanephrine; NE = norepinephrine. Conditions: 10% F-60, temperature programming at 1.5°/min.

3.3.4. Condensation products

Umeh[554] separated and determined the isomers of aniline and toluidine, after their reaction with formic acid, in the form of formanilide and formtoluidide. Moffat and Horning[400] used condensation with pentafluorobenzaldehyde in order to prepare the derivative of phenethylamine, which shows the highest sensitivity in detection with the ECD; it was possible to determine as little as 10 pg of the amine.

3.3.5. Isothiocyanates

These compound are prepared by the reaction of the primary amine group with carbon disulphide and have good chromatographic properties. Brandenberger and Hellbach[65] resolved amphetamine and its methyl derivative by this means and applied the method to the determination of these substances in urine. The reaction proceeds as follows:



These derivatives were studied with various biogenic amines by Narasimhachari and Vouros[420,421], who published their chromatographic and mass-spectrometric data.

3.3.6. Other derivatives

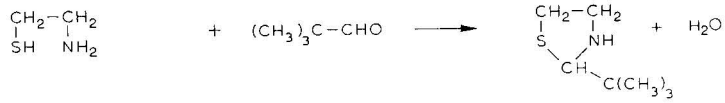
Gejvall and co-workers[213,471] separated and determined several low-molecular-weight amines after their conversion into urethanes by reaction with diethyl dicarbonate:

$$R\text{–}NH_2 + C_2H_5O\text{–}CO\text{–}O\text{–}CO\text{–}OC_2H_5 \rightarrow R\text{–}NH\text{–}CO\text{–}OC_2H_5 + CO_2 + \\ + C_2H_5OH$$

Dee[137] determined hydrazine and methylhydrazine as the corresponding pyrazoles after their reaction with acetylacetone. Neurath and Lüttich[424] converted asymmetric hydrazines into 5-nitro-2-hydroxybenzal derivatives. Sen[497] determined nitrosamines after their oxidation to nitramines with hydrogen peroxide. Frére and Verly[189] oxidized amines in aqueous solutions with an iodate and separated chromatographically the aldehydes produced. Hucker and Miller[283] chromatographed tertiary amines and compared their properties with those from the Hofmann reaction. These products are separated much better than the free amines and no tailing of the peaks occurs. Jenden and co-workers[244,295,296] analyzed quaternary choline and acetylcholine after their demethylation with benzene thiolate in butanol at 80°, injecting the tertiary amine into the gas chromatograph. The method was applied to the determination of choline and acetylcholine in biological materials. In order to separate optical isomers, Beckett and Testa[40] used N-TFA-L-prolyl derivatives prepared by the reaction of amphetamines with N-TFA-L-prolyl chloride. Corbin and Rogers[115] resolved the TFA, PFP and HFB derivatives of enantiomers of secondary amines on an optically active stationary phase, the ureide of L-valine isopropyl ester.

3.4. Sulphur compounds

Thiols require more drastic conditions for silylation. 1-Butanethiol does not react with an HMDS–TMCS mixture even under reflux, and the sodium or lead salts of saturated thiols have to be used in order to prepare their TMS thioethers[348]. Jellum *et al.*[294] analyzed cystine, cysteamine and other biologically important amines in the form of the thiazolidines and neopentylidines, after their reaction with pivaldehyde:



Barron and Mooney[36] chromatographed thioesters after their reduction with sodium borohydride. The alcohols produced were extracted and determined either directly or after their derivatization. Burchfield *et al.*[83] determined 4,4′-diaminophenyl sulphone and 4-acetamidophenyl-4′-aminophenyl sulphone after their conversion into iodo or bromo derivatives. N,N-Dialkyldithiocarbamates were chromatographed after their S-alkylation with diazomethane. Ethyl and propyl esters were prepared by reaction with iodoethane and 1-iodopropane, respectively[431].

3.5. Carboxylic acids

The direct analysis of free carboxylic acids by GC has been studied thoroughly and the results were good[529]. However, the presence of a carboxyl group in the molecule of the solute necessitates either a modification of the column packing or the use of a special technique in order to prevent the spurious adsorption of solute in the column[425]. The carboxyl group can be blocked simply and efficiently by esterification, and the chromatographic separation of the esters does not present serious difficulties. With substituted carboxylic acids, such as hydroxy and keto acids (amino acids are discussed separately), the situation is further complicated by the presence of the other functional groups and chemical derivatization of such compounds is essential. Chemical conversion of the acid into another compound is used only rarely, in special instances. For instance, 3-methoxy-4-hydroxymandelic acid can be chromatographed after its reduction and conversion into the vanilyl TFA ester[139]. A detailed study on the GC of carboxylic acids and their derivatives was described by Supina[529].

3.5.1. Esters

Owing to the ease of their preparation and their good chromatographic properties, these derivatives are widely used and have been studied in great detail. Methyl esters are the most commonly used esters. With carboxylic acids, methylation has been carried out with diazomethane[399,456,487,488], methanolic hydrochloric acid[282,390,526], methanolic boron trifluoride[9,20,394,395,410] and boron trichloride[67], and by the pyrolytic decomposition of tetramethylammonium salts[31,152,153,466]. In the esterification of keto acids (Krebs cycle), the reactions are complicated by the by-products

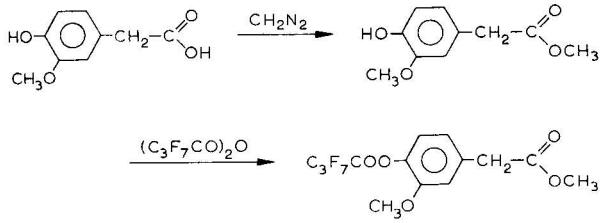
from the methylation of the enolized keto group[508]. When employing this method, Estes and Bachmann[172] observed decomposition of the derivatives in the column. Hautala and Weaver[256] employed boron trifluoride–methanol mixture for the esterification of the acids of Krebs' cycle acids, whereby pyruvic acid is converted into methyl 2,2-dimethoxypropionate. Felder *et al.*[179] used N,N'-dicyclohexylcarbodiimide in methanol for the preparation of the methyl esters of halobenzoic acids.

In general, it can be stated that methyl esters are suitable for the GC of higher fatty acids, *e.g.*, those produced by the hydrolysis of fats[298,359,361]. The methyl esters of lower fatty acids are volatile and may be subject to losses during their preparation. In order to perform the simultaneous determination of glycerol and fatty acids in the hydrolyzate of fats, Mason and co-workers[376,378] added 2,2-dimethoxypropane to the reaction mixture, thus converting glycerol into isopropylideneglycerol, which had good chromatographic properties. Some workers have employed higher alcohols for the esterification of lower acids. Dummel and Kun[155] described ethyl esters, Karmen[312] used 2-chloroethyl esters and Smith and Tsai[519] used trichloroethyl esters, which afford a selective analysis with the use of a halogen-sensitive detector. Appleby and Mayne[22] studied *n*-propyl esters and Thenot *et al.*[548] described their preparation by reaction with N,N-dimethylformamide dipropylacetal.

Butyl esters are prepared in a similar manner to methyl esters. Bezard and Bugant[49] employed butanol plus 2% of sulphuric acid and other workers[27,215,389] have used butanol saturated with hydrogen chloride and/or butanol plus boron trifluoride[341]. Wilcox[587] prepared the higher alkyl esters of hydroxybenzoic acids by reaction with diazoalkanes, catalyzed with 0.007% of boron trifluoride. In the absence of boron trifluoride, multiple peaks appeared in the chromatogram.

Benzyl esters[118,332] and *p*-substituted benzyl esters[578] have been used with short-chain fatty acids. Craig *et al.*[122] analyzed acids up to C_9 as their butyl and phenacetyl esters. Umeh[556] described the separation of the *p*-bromophenacyl and *p*-phenylphenacyl esters of C_2–C_{10} acids. In order to increase the sensitivity of analysis, Kawahara[319] used pentafluorobenzyl esters.

The methylation of phenolic hydroxy acids with diazomethane does not give uniform products owing to incomplete methylation of the hydroxy group[589]. Some workers[275,278,313] used trimethylsilylation in order to derivatize free phenolic and alcoholic groups. Sjöquist and Änggärd[512] chromatographed homovanillic acid after its conversion into the methyl ester of the heptafluorobutyrate:



Similarly, Dziedzic *et al.*[160] employed the hexafluoroisopropyl ester of the trifluoroacetate and obtained a high sensitivity with the use of an ECD. Anthranilic acid has been analyzed as the methyl ester of its trifluoroacetate[264].

3.5.2. Trimethylsilyl derivatives

With carboxylic acids, silylation agents react both with the carboxyl group

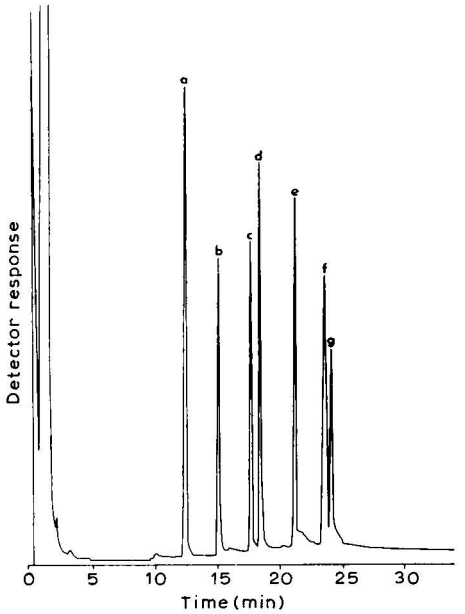


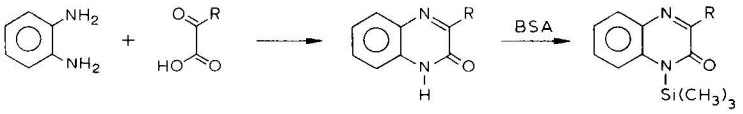
Fig. 6. Chromatogram of silylated phenolic acids[408]. a, *p*-Hydroxybenzoic acid; b, vanillic acid; c, syringic acid; d, coumaric acid; e, ferullic acid; f, sinapic acid; g, *n*-docosane. Conditions: 3% UCW-98 on Chromosorb W HP; temperature programming at 6°/min, initial temperature 100°.

proper and with other groups present in the molecule[449]. The use of these derivatives is especially advantageous in instances when acids are determined in the presence of other compounds that can also be silylated. Thus, Tallent and Kleiman[534] used BSA for the silylation of the hydrolyzate from lipids. The TMS esters showed good chromatographic properties and were separated from the ether of glycerol. Sato and Von Rudloff[484] determined saccharides and alcohols together with acids in the benzene extract from heart wood. In addition to the analysis of aliphatic acids[277,303,514,601], this method has been particularly useful with hydroxy acids[170,311,443,594] and polyhydroxy acids[446,504]. The complete silylation of all of the hydroxy groups present usually requires the catalytic action of TMCS. Aromatic acids can be converted by HMDS–TMCS into silyl derivatives only by refluxing the reaction mixture in toluene[317]. Better results were achieved by the use of BSA as a silylation agent[119]. The reaction is more rapid and the products are more uniform, even with phenolic acids[130,408] (Fig. 6).

If indolecarboxylic acids are to be silylated, it is necessary to use a stronger silylation agent in order to silylate the indolic nitrogen also. Good results are obtained with BSTFA in acetonitrile, and the separation of the TMS derivatives is better than that with the methyl esters[227].

In addition to the above TMS methyl ester derivatives, other combinations have also been used for the conversion of substitution derivatives of acids into volatile compounds. With Krebs' cycle acids, the use of TMS derivatives was only partially successful[472]. The ketonic group produces multiple peaks of keto acids (owing to enolization and decomposition in the column) and has to be blocked in a preliminarily stage[129]. The conversion of the ketonic group into an oxime with hydroxylamine and

subsequent silylation give derivatives that can be analyzed satisfactorily[271]. However, methoxime-TMS derivatives have been used more frequently[16,276,308]. Chalmers and Watts[96] have also applied ethyl and benzyl oximes successfully. Interesting derivatives were introduced by Hoffman and Killinger[267], which were later employed by other workers[192]. Keto acids are converted by reaction with aromatic *o*-diamines into the derivatives of chinoxalone, which, after silylation, can be chromatographed satisfactorily:



Scott[492] employed chlorosulphonic acid in order to block the hydroxy groups of hydroxy and hydroxyphenolic acids before their silylation with BSTFA. A better separation, compared with the TMS ester–ethers, was obtained with dihydroxy compounds in particular.

3.5.3. Dinitrophenylhydrazones

These derivatives can be used with acids that contain a carbonyl group, particularly with keto acids. Their importance is based mainly on the possibility of carrying out a preliminary separation of keto acids from the sample. Earlier, the chromatographic analysis was carried out after methylation and the liberation of the methyl esters from the 2,4-DNPH by ozonolysis[469]. However, the peaks of the by-products often overlap with the peaks of the keto acid esters and interfere with the analysis. Kallio and Linko[309] therefore chromatographed the 2,4-dinitrophenylhydrazones of methyl esters of keto acids directly. However, in this instance also, extra peaks due to isomeric derivatives occurred.

3.5.4. Anilides and toluidides

Umeh[555] used these derivatives successfully with lower carboxylic acids and described the conditions for their separation. The derivatives of formic acid can also be used for the separation and identification of the isomers of aniline and toluidine[554].

3.5.5. Cyclic boronates

n-Butyl boronates, which are usually used with difunctional compounds, can also be used with hydroxy and keto acids[75]. The advantage of these derivatives lies in the simultaneous separation of other difunctional compounds, such as diols, ketols and hydroxylamines.

3.5.6. Separation of optical isomers

As observed by Ackman *et al.*[1] and Annett[18], the enantiomers of hydroxy acids can be separated satisfactorily as their L-menthyl esters. Hammarström and Hamberg[239] used methyl ester D-phenyl propionates for the separation of the diastereoisomers of 3-, 15-, 16- and 17-hydroxyoctadecanoates. Poorer results were obtained with 2-, 14-, 4-, 7- and 13-hydroxyoctadecanoic acids.

3.6. Amino acids*

The low volatility of amino acids, caused by the presence of a carboxylic and an amine group in the molecule, renders the GC analysis of free acids impossible. Compared with conventional analysis on ion exchangers, the use of chemical derivatives of amino acids has three substantial advantages: (1) the possibility of determining 10^{-4}–10^{-5} times smaller amounts of amino acids; (2) the possibility of shortening the time of analysis to as little as half an hour; and (3) the possibility of utilizing the instrumentation for other purposes. Nevertheless, the full utilization of these advantages is hindered by two general difficulties: (1) the quantitative preparation of suitable derivatives of all amino acids; and (2) the choice of a suitable selective sorbent on which all the derivatives will be well separated.

The difficulty in selecting a suitable derivative is due largely to the widely varying structures and chemical properties of amino acids. Difficulties are encountered particularly in the preparation of volatile derivatives of arginine, histidine and tryptophan. The acylation of the guanidine group of arginine and the imidazole group of histidine is complicated by the formation of salts in the strongly acidic medium that is necessary for the reaction. Also, the indolic nitrogen of tryptophan is difficult to acylate, and although the second centre of basicity is acylated easily, the monoacyl derivative produced is eluted from the column only at high temperatures. Lysine also needs a strongly acidic medium for the acylation of its second amine group. The derivatization of cysteine has to be carried out in an inert atmosphere, as cystine, which is produced on oxidation, forms a high-boiling derivative that is difficult to elute.

Other methods of blocking polar groups (*e.g.*, the preparation of TMS derivatives, dinitrophenylhydrazones and diisopropyl derivatives) are usually suitable only for a limited number of amino acids and/or in special instances. Detailed papers dealing with the problems of the preparation and properties of volatile derivatives of amino acids and with the application of the methods to biological samples have been published by Blau[53] and McBride and Klingman[382].

3.6.1. Acyl alkyl ester derivatives

These compounds are the most commonly used derivatives in the GC of amino acids. Different workers have used various combinations of the acyl and alkyl moieties; N-trifluoroacetyl-*n*-butyl esters proved to be most suitable. The possibility of using these derivatives in GC was demonstrated by Zomzely *et al.*[602] in 1962 and later by Lamkin and Gehrke[342]. Mussini and Marcucci[416] employed diazobutane for the esterification of amino acids; a more common method for the preparation of esters of higher alcohols is to carry out the reaction in the presence of anhydrous hydrogen chloride. With amino acids, this reaction is complicated by their low solubility in higher alcohols. Therefore, Blau and Darbre[56] dissolved the sample in a small amount of trifluoroacetic acid before the addition of the alcohols, and Stalling *et al.*[524] prepared butyl esters by transesterification of methyl esters, thus utilizing their solubility in butanol. Roach and Gehrke[148] successfully employed 3 *N* hydrochloric acid in *n*-butanol, shortening the time of reaction to 15 min.

* A more extensive treatment of this topic was given by Hušek and Macek, *J. Chromatogr.*, 113 (1975) 139.

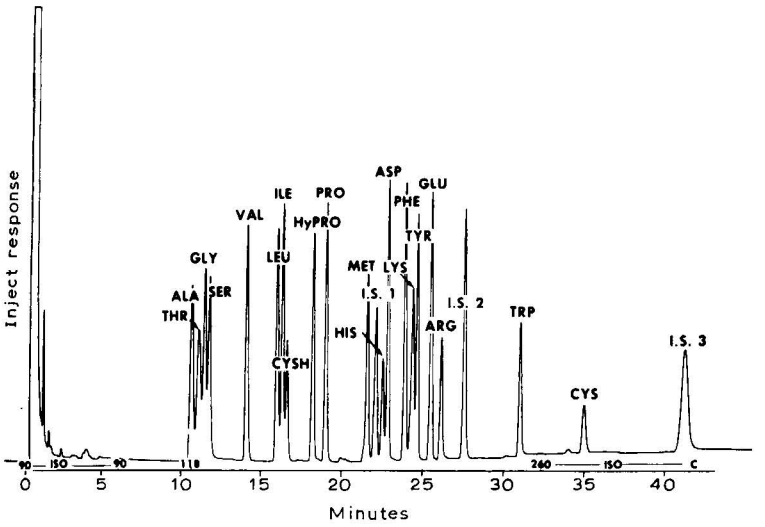


Fig. 7. Chromatogram of N-TFA-*n*-butyl esters of natural amino acids[210]. Conditions: glass column, 2.5 m × 2 mm I.D.; 10% Apiezon M on Chromosorb W HP, 80–100 mesh; temperature programming at 6°/min; initial temperature 90°. Internal standards (I.S.): 1, ornithine; 2, tranexamic acid; 3, *n*-butyl stearate.

The quantitative aspects of the preparation and determination of N-TFA-butyl esters of amino acids have been dealt with in several papers by Gehrke and co-workers[207,208,212] and McBride and Klingman[383]. As a result of their investigations, methods were developed for the macro, micro[201] and submicro[211] determinations of natural amino acids. The difficulties associated with the separation of the derivatives were obviated by employing two columns in series and mixed stationary phases[147,149,202,603]. More recently, Gehrke and Takeda[210] described the conditions for the separation of the derivatives of 20 amino acids on a single column (Fig. 7). Raulin *et al.*[464] described the application of this method to the analysis of a further 18 amino acids that are not present in proteins. Other workers have applied the method to the determination of amino acids in water[576], plasma[441] and other biological samples[92,260,339]. These derivatives can also be used for the resolution of optical antipodes[216,455,463], but with the *n*-butanol being replaced by *sec.*-butanol.

Compared with the above derivatives, N-trifluoroacetyl methyl esters of amino acids have the advantage of easier preparation, as amino acids are sufficiently soluble in the methanol–hydrochloric acid mixture, and no difficulties have been encountered in the derivatization[39,125]. However, Islam and Darbre[287] noticed that the methyl esters have a high volatility, which can cause losses during preparation, and developed an instrument[544] that enables the risk of such losses to be minimized. Although N-TFA-methyl esters have been used for the identification[54,370,584] and determination[111,231] of amino acids by some workers, these derivatives have not found wide application.

N-Trifluoroacetyl amyl esters were studied by Teuwissen *et al.*[545] and have been described in a number of papers by Darbre and Blau[55,56,131,132]. Compared with the methyl and butyl derivatives, amyl esters are less volatile and more stable, which

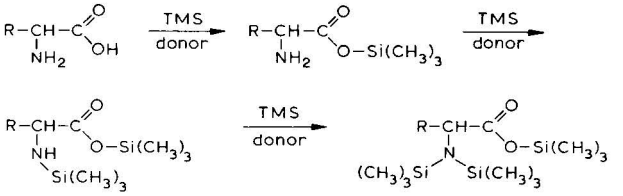
results in minimal losses during their preparation and analysis. However, the analysis is complicated by the incomplete separation of some of these derivatives, which restricts the application of the method; so far, this method has been used with only a limited number of amino acids[133].

Other combinations of the acyl and alkyl moieties have also been studied. The aims of the studies were to find more stable derivatives with shorter retention times that are easy to prepare and to enhance the sensitivity of analysis by employing highly fluorinated acyl anhydrides. Coulter and Hann[113] described the GC of N-acetyl-*n*-propyl esters of amino acids, and Pollock[454] studied pentafluoropropionyl and heptafluorobutyryl butyl esters, which have retention times that are as much as 35% lower than those of TFA-butyl esters and provide for selective detection with the ECD. Recently, remarkable results were obtained by Moss and Lambert[413] and Jönsson *et al.*[304] with N-HFB-*n*-propyl esters and by Zanetta and Vincendon[598] with HFB-isoamyl esters of amino acids. After esterifying the carboxyl group, Halpern *et al.*[234] protected the amine group by converting it into the N-thiocarbonyl group and achieved a good separation of the derivatives.

In addition to the above N-TFA-*sec.*-butyl esters, other derivatives have been employed for the separation of optical antipodes. In particular, N-TFA- and N-PFP-isopropyl esters[116,435–438] and N-TFA-2-octyl esters[216] have found application in this respect. Bonner[60] used N-TFA-S-prolyl methyl esters for the separation of *R*- and *S*-enantiomers of leucine. Fu and Mak[193,194] published a comprehensive comparison of different N-acyl alkyl derivatives of amino acids and the effect of the substituents on their chromatographic behaviour, and presented the retention data.

3.6.2. Trimethylsilyl derivatives

These derivatives were used in the GC of amino acids as early as 1960 by Birkofer and Ritter[51] and in 1961 by Rühlman[479] and Rühlman and Giesecke[480]. However, further work met with problems associated with the non-uniformity and instability of the products of silylation. The presence of different functional groups in the molecules of amino acids, displaying different reactivities toward silylation agents, results in the formation of different products, depending on the strength of the silylation agent employed and on the reaction conditions:



It was not until 1965 that Smith and co-workers[379,517] began to study the TMS derivatives of amino acids more systematically, investigating the efficiency of the individual silylating agents on model compounds. TMS-amines, particularly TMSDEA, were recommended as suitable reagents. Amides (BSA) give lower yields, and TMS-imidazole does not silylate amino acids[518]. Similar results were obtained by Klebe *et al.*[331], who introduced BSA as a strong silylation agent. The silyl derivatives that they prepared except for that of arginine, gave single sharp peaks, but decomposed

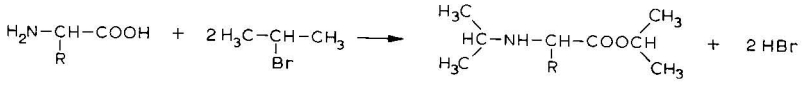
in the column. In addition, the mono-TMS-acetamide produced as a by-product during the reaction interfered with the derivatives of glycine and alanine. Mori *et al.*[404] applied BSA to the analysis of the products of the hydrolysis of copolyamides and determined the TMS derivatives of amino acids, diacids and diamines in their mixtures. In a similar manner, Caldwell and Tappel[90] separated sulpho- and selenoamino acids and the products of their oxidation.

Sharokhi and Gehrke[498] introduced the silylating agent bis-TMS-trifluoroacetamide (BSTFA) and Gehrke and co-workers[205,206] studied the conditions for the silylation of all natural amino acids. The higher volatility of the mono-TMS-trifluoroacetamide produced in the reaction permits the separation of this by-product from the derivatives of lower amino acids. However, the structure of the derivatives produced is strongly dependent on the reaction conditions[247]. For instance, glycine gives a single peak of the monosubstituted derivative when employing a solvent of low polarity (hexane, methylene chloride, chloroform, 1,2-dichloroethane) and two peaks of the di- and trisubstituted derivatives in more polar solvents[204]. Bergström *et al.*[46] studied the structure of the silyl derivatives of amino acids, prepared with BSTFA in the absence of a solvent. In this instance, glycine gave the mono-, di- and trisubstituted derivatives, lysine gave the tri- and tetrasubstituted derivatives and 6-aminocaproic acid gave the di- and trisubstituted derivatives.

In spite of the above difficulties, silyl derivatives have been employed in the determination of amino acids in biological samples[53,205,353]. Poclington[453] determined amino acids in the water of the Atlantic Ocean in this way. Dabrowiak and Cooke[127] separated the enantiomers of threonine as the TMS-N-TFA-D-(L)-prolyl methyl esters.

3.6.3. Diisopropyl derivatives

Pettitt and Stouffer[448] recently introduced promising derivatives. The reaction with 2-bromopropane leads to the isopropyl esters, but, at the same time, the isopropyl group is attached to the amine group:



The ε-amine group of lysine, the phenolic group of thyrosine and the thiol group of cysteine are also blocked. Only the hydroxy group of hydroxyproline is unreactive. Thus, the protection of almost all of the polar groups that occur in amino acids can be achieved by this single-step reaction. However, the different reactivities of different groups have an appreciable effect on the uniformity of the products and makes quantitative analysis difficult to perform[57]. Blessington and Fiagbe[58] used these derivatives for the identification of amino acids and amines in urine.

3.6.4. Dinitrophenyl derivatives

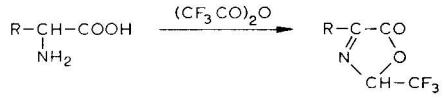
These derivatives can be used only for a limited number of amino acids, as the derivatives are not stable[452]. The carboxyl group is usually esterified with diazomethane[284] or by a mixture of methanol and thionyl chloride[398]. Although this method cannot be applied to all amino acids, it is useful for the determination of terminal amino acids in proteins[347,473].

3.6.5. Cyclic derivatives

The two functional groups in the molecule of an amino acid provide for the preparation of cyclic derivatives, the simplest of which are diketopiperazines[53]:

However, as they have two amide groups in the molecule, these compounds are not very volatile and also have other properties[535] that make them unsuitable for gas chromatography. Much better properties are displayed by phenylthiohydantoins, which were chromatographed successfully by Eriksson and Sjöquist[167] and Pisano *et al.*[452]. As these compounds are the final products of the Edman degradation of peptides, they provide for the rapid sequentional analysis of amino acids[344]. Nevertheless, these derivatives are not ideal as they require too high temperatures for elution (as high as 290°) and the derivatives of serine, threonine and cystine are unstable[53]. Some workers tried to overcome these difficulties by the preparation of methylthiohydantoins[26,174,451], N-TMS-phenylthiohydantoins[248,343,462] and N-TFA-phenylthiohydantoins[66,467].

The preparation of 2-trifluoromethyl-4-substituted oxazolones and their separation on a capillary column was described by Weygand[583]; recently, these derivatives were studied by Grahl-Nielsen and Solheim[220]. The derivatives are prepared by refluxing amino acids with TFA anhydride:



Some amino acids (serine and methionine, for instance) do not give these derivatives.

3.7. Thyroid hormones

As with amino acids, thyroid hormones also contain polar groups in the molecule, *i.e.*, carboxyl, hydroxy and amino groups, which have to be blocked prior to GC. Richards and Mason[465] esterified the carboxyl group by reaction with TFA anhydride and converted the ester into the N,O-bis-TFA methyl ester. Unfortunately, the authors did not give any quantitative data on the yield of the reaction and the sensitivity of analysis.

All of the polar groups in thyroid hormones can be efficiently blocked by single-step trimethylsilylation. Sharokhi and Gehrke[499] used both HMDS–TMCS and BSA in acetonitrile for this purpose. Good yields were obtained in both instances and a single peak for each substance appeared in the chromatogram. Other workers[8,29] obtained similar results. Jaakonmäki and co-workers[288,527] tried to obviate the instability of TMS derivatives towards humidity by preparing N,O-dipivalyl methyl esters, which are stable. They can be pre-purified by thin-layer chromatography[571] and give a sufficiently sensitive analysis with the use of an ECD.

3.8. Steroids

The choice and preparation of derivatives for the analysis of steroid compounds

are also complicated by the presence of different functional groups in the molecule. Four main polar groups, alcoholic, phenolic, ketonic and carboxylic, are encountered with steroids that occur in nature. Although some steroids are amenable to GC[428,429], chemical blocking of the polar groups is usually advantageous and sometimes essential. It improves the symmetry of the GC zones and the quantitativeness of their elution and usually increases markedly the volatility and stability of these compounds. The derivatization also permits some steric differences in steroids to be distinguished. An example is the separation of the epimers testosterone and epitestosterone, which can be well separated only after their conversion into TMS or acetyl derivatives[564]. The different retention characteristics of slightly differing steroids can be utilized for identification purposes[99,334]. Differences in the rate of derivative formation, caused by differences in steric hindrance[68], can be utilized in the same way. The selective formation of derivatives can be utilized advantageously for the removal of non-specific compounds from the sample[72,121]. If sub-nanogram amounts of steroids are to be analyzed, such as steroid hormones in the blood, for example, the preparation of derivatives that attract electrons is useful. Several workers have published comprehensive papers comparing the chromatographic behaviour of different derivatives of steroids and listing their retention data[164,229,406,564]. Baillie *et al.*[33] discussed the preparation and properties of the derivatives of corticosteroids and Knights[333] applied various derivatization methods to plant sterols. The separation of derivatives of steroids on a capillary column was demonstrated by Novotný and Zlatkis[429]. Berthou *et al.*[48] chromatographed derivatives of urinary steroids.

3.8.1. Silyl derivatives

The most commonly employed reaction for the preparation of volatile derivatives of steroids is silylation. Luukkainen *et al.*[367] were the first workers to utilize silyl derivatives of steroids in GC. A number of silylating agents have since been developed, TMCS, HMDS, TMSDEA and BSA being the most commonly used. When preparing the derivatives, it is necessary to observe the usual conditions of silylation reactions, which is difficult with biological samples and may lead to errors. A detailed discussion of these problems, together with valuable comments, has been presented by Pierce[449].

The currently most commonly used reagent, BSA, is so reactive that it converts all of the unhindered hydroxy groups (3-, 16-, *sec.*-17-, 20-, 21-) of steroids into TMS ethers without catalysis in several hours[272]. For the somewhat hindered 11β-hydroxy group, catalysis with TMCS is required in order to convert it into the TMS derivative[98]. The more powerful reagent BSTFA also reacts with the 11-hydroxy group, but the reaction proceeds slowly, so that the use of BSTFA without catalysis is not recommended. Strongly hindered groups (*e.g.*, 17α- in 17α,20α,21- or 17α,20β,21-triols) can be converted into TMS ethers only by using TMSIM in admixture with BSA–TMCS[98,481]. With digitoxigenin and related compounds, the reaction with TMSIM–BSA–TMCS mixture causes the lactone cycle to open and the enolized hydroxy group is also silylated[381], while HMDS silylates only the hydroxy groups of the skeleton[293,358]. Cowley *et al.*[120] used HMDS–TMCS–pyridine mixtures and studied the effect of the amount of solvent on the uniformity of the products. However, the use of this mixture causes complications due to the formation of a precipitate of ammonium salts[481].

In biological applications, it is rarely possible to use TMS derivatives

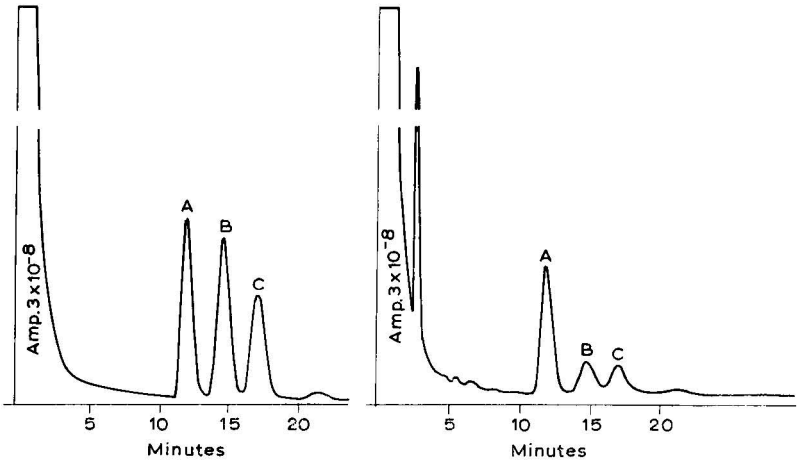


Fig. 8. Chromatograms of TMS derivatives of urinary 17-keto-steroids[47]. Left: model mixture of androstene (A), etiocholanolone (B) and dehydroepiandrosterone (C). Right: urine extract. Conditions: glass column, 1.8 m × 6 mm I.D.; 2% GE XE-60 on silanized Gas-Chrom P; temperature 225°, isothermal.

alone[76,101,230,510] (Fig. 8) and it is usually necessary to block other groups that are present. The use of oximes (see later) has proved useful[97,273]; Nambara and Bae[419] even employed a preliminary epoxidation of androstenes. The carboxyl group of bile acids has to be converted into its methyl ester by reaction with diazomethane prior to silylation[166]. Some suprarenal-gland hormones can also be determined in a similar way after their preliminary oxidation to 17α-carboxylic acids with periodic acid[330,392]. Fisher *et al.*[183] noted a marked instability of the TMS derivatives of vitamin D and related compounds. However, these difficulties do not occur with other steroids under anhydrous conditions. Chloro-, bromo- and iodomethyl dimethylsilyl ethers have also been described, but they have not found wide application[558,560]. They were studied systematically also by Eaborn and co-workers[162,219,550], who demonstrated their application to the determination of urinary steroids[551]. The derivatives yield a higher response with the use of the ECD[161,549] and characteristic mass spectra in combined GC–MS[100]. Morgan and Poole[402] employed trifluoropropyl, heptafluoropentyl and pentafluorophenyl dimethylsilyl derivatives for the selective determination of steroids from the group of ecdysones.

3.8.2. Acyl derivatives

Although the most important derivatives of this type are halogenated acyl derivatives, acetates of corticosteroids and sterols[71,427] have also been used successfully. Mougey *et al.*[414] resolved the epimers testosterone and epitestosterone as their acetates.

Landowne and Lipsky[346] and Brownie *et al.*[80] described the chromatographic behaviour of different haloacetates of steroids. However, because of their higher volatility and higher response in detection with the ECD, heptafluorobutyrates have found more general application[95,140,265,577,590]. Wotiz and co-workers[107,595] used these derivatives in the determination of steroids in biological samples. In order to deter-

mine trace amounts of steroids, Nakagawa *et al.*[418] prepared perfluorooctanoates and Kirschner and Taylor[328] prepared hexafluorononanoates and eicosafluoroundecanoates. The response of these derivatives is more than twice that of HFB derivatives. The preparation of acyl derivatives of steroids is easy, only Δ^4-3-ketosteroids giving a non-uniform product because of the enolization of the carbonyl group[140]. With polyfunctional steroids, acyl derivatives are used in combination with other derivatives, particularly with oximes[274].

3.8.3. Siliconides and acetonides

These derivatives come into consideration with steroids that contain two *vicinal* hydroxy groups. The acetonide derivative was utilized for the GC determination of estriol by Adlercreutz *et al.*[2]. Acetonides were also employed in analysis of corticosteroids[30]. Kelly[321,322] described the GC of the siliconides of corticosteroids that have hydroxy groups in the 16α,17α- or 17α,21-positions.

3.8.4. Bismethylenedioxy derivatives and cyclic boronates

As with siliconides, these derivatives can also be prepared from steroids with a dihydroxyacetonic side-chain. Bismethylenedioxy derivatives[327] and boronates[19] have been used with corticosteroids. Brooks and Harvey[74] described cyclic boronates with 17,20-diol, 20,21-diol and 17,20,21-triol steroids. The other free hydroxy groups on the skeleton were blocked by trimethylsilylation or acetylation.

3.8.5. Oximes

These derivatives are employed for protecting the carbonyl group of ketosteroids. Most commonly used are methoximes[176], which are usually combined with other derivatives of the hydroxy group, particularly silyl derivatives[261,280,546,547]. Benzyloximes[141,142] and pentyloximes and butyloximes[32] in combination with TMS derivatives have also been described. They have higher retention times and are therefore well separated from the related hydroxy compounds and possess good properties for use in combined GC–MS.

3.8.6. Hydrazones

These derivatives are prepared for the same purpose as oximes. Their preparation has been described by VandenHeuvel and co-workers[562,563]. Hydrazones can be used with advantage to study structural differences in ketosteroids by means of retention data. Pentafluorophenylhydrazones have an appreciable affinity for electrons and provide for the sensitive detection of steroids with a ketonic group in plasma[25].

3.8.7. Methyl ethers

The application of the methyl ethers of phenolic steroids with the hydroxy group on the third carbon atom was described by Brown[79]. Clayton[110] extended the use of this method to a further 30 steroids (estrogens). A more common application of this method is its combination with acetylation[121,391] or trimethylsilylation[3,366] for the routine determination of estrogens by GC.

3.8.8. Ketals

Sarfaty and Fales[482] prepared these derivatives by the reaction of the ketone

(usually a halogenated acetone) with the steroidic alcohol and methylating with diazomethane the hemiketal produced. The derivatives are sufficiently stable and are not hydrolyzed, even in aqueous solutions, but the sensitivity of their detection with the ECD is lower than that with HFB esters.

3.9. Saccharides and related compounds

The molecules of carbohydrates contain, in addition to hydroxy groups, other polar groups, such as carbonyl, carboxyl and amine groups. Their volatility is very low, so that they cannot be chromatographed as such. The polyfunctionality of carbohydrates makes the quantitative preparation of a uniform product difficult, and often as many as four peaks result from a single compound, which complicates the identification of the individual compounds. Hence, with mixtures the chromatograms are very involved and their interpretation is difficult. Nonetheless, silyl derivatives and methyl ethers of saccharides and related compounds are used in GC. A detailed treatment of these problems and a number of valuable references can be found in the papers by Sloneker[515] and Wells *et al.*[581].

3.9.1. Methyl ethers

The separation of mono-, tri- and tetramethyl ethers of sugars was described as early as 1958 by McInnes *et al.*[388]. Their results were promising and pentoses were easily separated from each other and from hexoses. However, the separation of hexoses and their anomers was unsatisfactory. Whyte[586] described the GC of methylated sugars and hexuronic acids. Kircher[325] employed a specially adapted column and separated successfully both the α- and β-anomers of methyl pyranosides from methyl furanosides.

The problem of the choice of the stationary phase, the degree of methylation and the application of the method to different carbohydrates have been dealt with by several workers[12,187,357]. Anderle *et al.*[13] described the separation of the optical isomers of xylofuranosides after their conversion into methyl ethers. As expected, derivatives that have a smaller number of methoxy groups have longer retention times, so that some monomethyl ethers have such long retention times that they are completely unsuitable for GC[581]. Therefore, when applying this method to disaccharides and polysaccharides, the products of their methanolysis have to be further modified. Kircher[325] methylated the polysaccharides with methyl iodide, while Bishop and Cooper[52] reduced the monomethyl ethers of sugars to the corresponding polyalcohols and analyzed them as their pentaacetates. A combination of methyl ethers with other derivatives, such as acetates and nitriles, has been used with sugars[61,345] and related polyalcohols[105].

3.9.2. Silyl derivatives

The preparation of the TMS derivatives of carbohydrates, which are polyhydroxy compounds, does not present any serious difficulties and they are completely silylated under mild conditions. Sweeley *et al.*[532] developed a simple versatile method, in which the silylation of a model compound, methyl α-glucopyranoside, was accomplished with HMDS–TMCS (2:1) in pyridine, which silylates all of the hydroxy groups of the substrate under normal conditions. Unless the reaction proceeds to completion, multiple peaks occur. They achieved an excellent separation even of the individual

anomers and configurational isomers of pentoses, hexoses, disaccharides and others. A modification of this method has also been used successfully by other workers for the preparation and GC of the TMS derivatives of glucose[114], glycosides[196,259,324], hexoses[543], heptoses[430] and other sugars[291,307,352] and related polyalcohols[159,188,225] and their oxidation products[143]. With some stationary phases, the pyridine produces a large tailing peak that can overlap some of the peaks of the derivatives, thus interfering with the determination of the latter. Lehrfeld[355] described a procedure for the removal of the pyridine from the mixture after the reaction has been completed and its replacement with *n*-hexane. Weiss and Tambawala[580] further simplified the method and carried out the reaction directly in aqueous solution. In this instance, the success of the determination consists in adding such a large excess of the silylation agent that all of the water is effectively removed.

The use of other reagents suffers from difficulties due to the formation of anomers. BSA in pyridine causes the anomerization of hexoses, and the chromatogram of a single sugar can contain four or five peaks[449]. In order to obtain uniform products, some workers oxidized simple sugars to the corresponding acids and their 1,4-lactones were converted into TMS derivatives, which were subsequently analyzed[409,445]. TMS derivatives have also been used successfully in the analysis of amino sugars by GC[457]. However, when employing a weaker silylating agent, only the hydroxy groups are silylated and the amine group remains unchanged[533]. Therefore, Perry[444] blocked the amine groups of some amino sugars by preliminary acetylation with acetic anhydride. The complete silylation of the phosphates of sugars is usually carried out with stronger silylation agents (BSA + TMCS and others)[249,500].

Halomethyldimethylsilyl and dimethylsilyl derivatives of carbohydrates are prepared under conditions the same as those for TMS ethers. In GC analysis, the halo compounds have retention times that are several times longer and their application is limited[531]. Dimethylsilyl ethers have retention times that are half of those of TMS derivatives and can be employed with advantage mainly with oligosaccharides[10].

3.9.3. Acyl derivatives

Acyl derivatives are used mainly with polyalcohols and sugars after their preliminary reduction. The method was introduced by Gunner *et al.*[228] and further developed by Sawardeker *et al.*[486]. Reduction with sodium borohydride and acetylation with acetic anhydride proceed quantitatively. Potential difficulties stem from the borate produced, which interferes in the acetylation, and from the pyridine solvent, which produces tailing peaks. Albersheim *et al.*[4] employed acetyl derivatives for the determination of sugars in cellular walls. Lehnhardt and Winzler[354] described the determination of sugars in glycoproteins (Fig. 9). Employing this method, Griggs *et al.*[226] determined neutral and amino sugars present together in mucins.

Excellent chromatographic properties are displayed by the acetates of aldonitriles of sugars, which are prepared by the reaction of sugars with hydroxylammonium chloride. The oxime produced is acetylated without its isolation and dehydrated to the nitrile by warming it in pyridine and acetic anhydride. Varma *et al.*[565] noted the long-term stability of these derivatives and applied the method to the analysis of natural material. They also analyzed in this way hexosamines after their deamination[232].

Vilkas *et al.*[569] described the successful separation of the TFA esters of mono-, di- and trisaccharides. The derivatives were prepared directly by the reaction of TFA

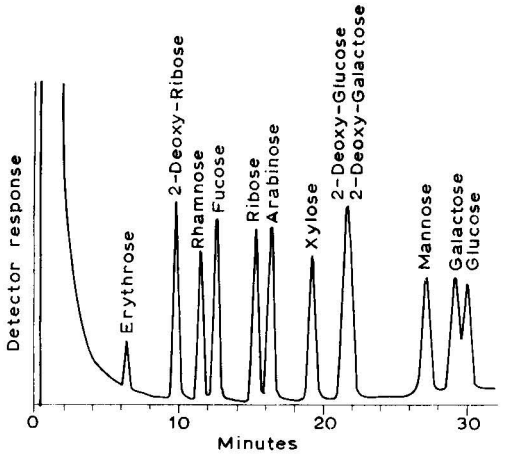


Fig. 9. Chromatogram of a mixture of sugars after their reduction and acetylation[354]. Conditions: glass column, 1.83 m × 4 mm I.D.; 0.75% Hi-EFF-1 BP, 0.25% EGSS-X and 0.1% 144-B (phenyldiethanolamine) on Gas-Chrom Q, 60–80 mesh; temperature programming at 1.3°/min, initial temperature 160°.

anhydride in acetonitrile with the carbohydrate. These derivatives are sufficiently thermostable and more volatile than TMS derivatives. In the acylation of amino sugars, the amine group is also blocked, which usually does not occur in silylation[597]. The application of these derivatives to sugars after their reduction to the corresponding alcohols was studied by Imanari *et al.*[286]. Anderle and Kováč[11] determined in this way mono-O-methyl-D-glucose after its reduction to monomethylglucitol and subsequent trifluoroacetylation.

3.9.4. Acetals, ketals and other derivatives

Kircher[325] described the successful GC of 4,6-O-ethylidene-D-glucose-1,2,3-O-triacetate. Adequate volatility for GC is also displayed by 1,2- and 5,6-O-isopropylidene derivatives. The chromatographic separation of these derivatives on a preparative scale was studied with 5- and 6-deoxyglucopyranose by Hedgley *et al.*[258]. A detailed study of the separation of acetal and ketal derivatives with free hydroxy groups or with the latter groups substituted with acetyl, benzyl, benzoyl, methyl or toluene-*p*-sulphonyl groups was published by Jones *et al.*[299].

3.10. Bases of nucleic acids, nucleosides and nucleotides

The direct GC analysis of the components of nucleic acids is rendered impossible by the presence of hydroxy and amine groups bound to the pyridine or purine core. Miles and Fales[396] used a combination of acetyl, methyl and isopropylidene derivatives in order to block the polar groups. They achieved a good separation of the products, but the peaks were asymmetric and showed considerable tailing. MacGee[368] studied the methyl derivatives and employed them for the determination of the ratios of the bases in nucleic acids. They were prepared by the thermal decomposition of tetramethylammonium salts in the injection port. The quantitative analysis

is hindered by the multiplicity of the products (there are as many as four peaks for adenine) and by the fact that different compounds may give the same derivative. For instance, the total methylation of xanthine, theobromine and theophylline results in caffeine in each instance.

A much wider use has been found for trimethylsilyl derivatives in the GC of nucleosides. Hancock and Coleman[243] prepared these derivatives by reaction with HMDS and TMCS in pyridine and obtained multiple products and asymmetric peaks. Later, Hancock[240] described the successful analysis of adenosine and showed that the TMS derivatives can be used for quantitative work. In a series of papers, Hashizume and Sasaki[251,254,483] described the GC separation of the TMS derivatives of some ribonucleotides and purine and pyrimidine bases. Employing phenanthrene as an internal standard, they obtained quantitative data for five main bases and demonstrated the application of the method to the determination of the ratio of bases in RNA and DNA.

Gehrke and co-workers[203,209] used BSA in acetonitrile as the silylating agent. They found optimum conditions for the preparation of uniform products; only cytosine and 3-methylcytosine gave two TMS derivatives. The five main bases were determined at the microgram level with a relative error of 3%. Further papers dealing with the GC of the derivatives of nucleosides are those of Hancock[241], Jacobson *et al.*[290] and Butts[85]. In the silylation of the derivatives of adenosine[241], small amounts of tetra-TMS derivatives were produced in addition to the stable tri-TMS derivatives produced by the silylation of the saccharide component. Jacobson *et al.*[290] successfully prepared TMS derivatives of guanosine and cytidine, which are among the nucleosides most difficult to silylate. Butts[85] converted the amine groups of nucleosides into methoximes by reaction with methoxylamine hydrochloride prior to silylation. The derivatives had shorter retention times and gave more symmetrical peaks than the TMS derivatives alone. The silylation of some less common nucleosides, *viz.*, those of dihydrouridine, pseudouridine, methylinosine, 6-thioguanosine and methylguanosine, was achieved with the use of BSTFA[242].

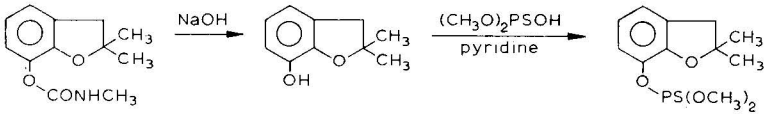
The application of TMS derivatives to the determination of the components of nucleic acids in their hydrolyzates has been described by different workers[203,254,340]. The hydrolysis is carried out with perchloric or formic acid and, after purifying the free bases on an ion exchanger, they are silylated and analyzed by GC. The method has been developed for work on the macro, semimicro and micro scales.

3.11. Insecticides and other pesticides

These substances comprise different types of chlorinated hydrocarbons, organophosphorus and -sulphur compounds, carbamates, heterocyclic derivatives and other substances. GC has proved very useful in the analysis of these compounds, and most pesticides have been analyzed successfully even in their free state[59]. The decomposition of chlorinated insecticides in the column and the presence of polar groups, especially hydroxy groups, in the molecules of some insecticides favours the preparation of chemical derivatives.

Chau and Cochrane[102,103] partially dechlorinated insecticides of the chlordane type with bases and converted them into uniform acetyl, TMS and/or *tert.*-butyl derivatives. This procedure gave single peaks with good shapes in the chromatogram

of each individual compound. Pionke *et al.*[450] analyzed directly insecticides that had been partially dechlorinated with potassium hydroxide, whereas endrin and dieldrin were saturated with hydrogen chloride prior to GC. The individual compounds were identified by chromatographing them after derivatization and in the free state on two stationary phases and comparing the retention data. Some workers blocked the phenolic and acidic groups of pesticides by acetylation[568], methylation with diazomethane[180] or trimethylsilylation[28,184]. In all instances the chromatographic properties were improved to such an extent that the chromatograms were amenable to quantitation. Diverse derivatives have been employed with carbamate insecticides. Bowman and Beroza[63] hydrolyzed carbamates with alkali-metal hydroxide to the corresponding phenols, which were further converted by reaction with dimethoxythiophosphate into the dimethoxythiophosphoryl derivative:



These derivatives display good properties for GC. Holden *et al.*[268] condensed the methylamine or dimethylamine group, liberated by hydrolysis, with 1-fluoro-2,4-dinitrobenzene and determined the resulting dinitroaniline with high precision using an ECD. Tatton and Wagstaffe[538] analyzed fungicides based on organomercury compounds. By reaction with an ethereal solution of diphenylthiocarbazone, the mercury present was converted into the dithizonates, which can be easily determined by GC.

3.12. Pharmaceuticals and drugs

The most suitable derivatives for use in the GC of these widely varying substances depend on the type of polar group present. Antibiotics are determined mostly as their TMS derivatives, which are useful because several different functional groups are often present in the molecule. Chloramphenicol and related antibiotics have been chromatographed by a number of workers[21,200,292,371]. As BSA does not give uniform products, HMDS–TMCS in pyridine is more suitable for the preparation of these derivatives. By employing a flame-ionization detector, concentrations as low as 0.2 μg/ml of these substances have been determined in biological materials. Tsuji and Robertson[552,553] have described a similar method for the determination of neomycins and attained high precision, despite the difficulty of hydrolyzing the derivatives. A number of penicillins have been analyzed in the same manner[266].

Vitamins comprise diverse chemical compounds and the range of the possible derivatives is also large[323]. Vitamin A has been chromatographed as its methyl and acetyl derivatives[156], but the instability of these derivatives necessitates the use of a special modification of the chromatographic column. The B vitamins are converted into acetyl, TFA or TMS derivatives prior to analysis[262,336,337]. With other vitamins, the polar groups in the molecule must also be blocked. Vitamin C was analyzed successfully as its TMS derivative[566] and vitamin D can be analyzed in a similar manner[575]. For the GC determination of vitamin E, conversion into the acetate was recommended[323].

Barbiturates can often be chromatographed, but they have long retention times and undergo strong adsorption in the column. These disadvantages do not occur with the methyl derivatives, which also give better resolution of the individual barbiturates. They are prepared either by reaction with a 10% solution of methanol saturated with potassium carbonate[374] or by pyrolysis of the tetramethylammonium[154,525] or trimethylanilinium[432] salts. Kowblansky *et al.*[338] resolved individual barbiturates and purines after their conversion with tetra-*n*-butylammonium hydroxide into the butyl derivatives. Parker *et al.*[434] identified barbiturates and other substances by utilizing the characteristic shifts in the retention times of the derivatives in comparison with those of the parent compounds.

Of the many other pharmaceuticals, we may mention the GC determination of narcotics as the acetyl and propionyl derivatives[415] and, of special interest, the determination of the analgesic pentazocine after its conversion into the pentafluorobutyryl ether[78] and of cambendazole after its silylation with BSA[561]. A more detailed survey of the GC analysis of pharmaceuticals and their derivatives has been presented by Kern *et al.*[323].

3.13. Anions of inorganic acids and related compounds

Anions of inorganic acids can be converted into volatile TMS derivatives and determined by GC. Following the work of Hashizume and Sasaki[252,253], who prepared the TMS derivatives of phosphates and glycerophosphates in very good yields by reaction with HMDS–TMCS, Butts[86] and Butts and Rainey[89] extended the method to other inorganic anions. Sodium and potassium salts are only slightly soluble in the derivatizing agent, thus giving low yields of TMS derivatives, and ammonium salts are more suitable. The above workers described the preparation and separation of the TMS derivatives of borates, carbonates, phosphates, arsenates, vanadates and other anions. Zinbo and Sherman[600] analyzed the TMS derivatives of phosphates by combined GC–MS. Wu *et al.*[596] identified the TMS esters of silicates by this means. Mattheuis *et al.*[380] determined trace amounts of phosphates in water after extraction of the ammonium salts with octanol–toluene and their subsequent trimethylsilylation. Boyden and Clift[64] used TMS derivatives in order to resolve phosphates and different mono-, di- and trialkyl-substituted phosphates. The TMS esters of aminoalkyl phosphates are not very stable and, therefore, the amine group must be blocked by converting it into an N-acetyl, isocyanate or similar derivatives[250].

Substituted anions of mineral acids can be determined by GC after their conversion into methyl esters. Taulli[539] resolved the isomers of hexadecene-1-sulphonic acid by the GC of their methyl esters, which were prepared with diazomethane. Various alkyl phosphates[128,246] have been determined in a similar manner.

Sulphonic acids are determined by GC as their methyl esters[45] or chlorides[263,326,440]. Nagai *et al.*[417] analyzed alkenyl and hydroxyalkyl sulphonates after their hydrogenation and conversion into sulphonyl chlorides. Parsons[439] showed that sulphonyl chlorides decompose in the column and are therefore unsuitable for GC separation. He converted sulphonyl chlorides into fluorides which are substantially more volatile and thermostable, by reaction with potassium fluoride. Shimoishi and Tôei[502] determined trace amounts of selenium in sulphuric acid by its oxidation to selenite and further conversion with 4-nitro-*o*-phenylenediamine into 5-nitropiaselenol.

The toluene extract of this derivative was chromatographed with high sensitivity with the use of an ECD. Srinivasan *et al.*[522] employed butylamine for the isolation of sulphates. After its isolation, the derivative is treated with an alkali-metal hydroxide and the butylamine liberated is determined by GC. MacGee and Allen[369] determined halide anions by converting their alkali-metal salts to tetraalkylammonium salts, which decompose to trialkylamines and alkyl halogenides upon injection into the gas chromatograph. In the determination of fluorides, the alkyl fluoride gives hydrogen fluoride and the corresponding olefin.

3.14. Miscellaneous

Durbin and Zlatkis[158] determined pyridine and quinoline bases after their reduction with hydrogen and conversion of the unsaturated compounds into pentafluoropropionates. The peaks were sufficiently symmetrical, and the sensitivity of analysis was increased by using an ECD. Ingle *et al.*[285] used the TMS ethers of the hydroxy derivatives of the above bases. TMS derivatives were also employed in the GC separation of pterins[362], coumarin and hydroxycoumarin[134]. Because of their higher volatility, TFA esters were also recommended for the GC of coumarin and its derivatives[135].

Slatter[513] determined diketopiperazines after their reaction with TFA anhydride. He resolved successfully all of the *cis*- and *trans*-isomers and diastereoisomers. Estas and Dumont[171] employed thermal degradation of tetramethylammonium salts for the methylation of hydantoins and their GC determination in biological samples. In comparison with other methods, this procedure has the advantages of specificity, sensitivity and speed of analysis. Glowacki *et al.*[218] determined 1,2-epoxides after their reaction with methanolic boron trifluoride or reduction with aluminium lithium hydride. In both instances, two products were obtained, but the ratio of their amounts was constant and the chromatograms could be evaluated quantitatively. Fioriti *et al.*[182] treated epoxyglycerides by reaction with ketones in the presence of boron trifluoride and chromatographed the derivatives of 1,3-dioxolane produced. Cyclopentanone was shown to be the most suitable starting material for the preparation of these derivatives. McDonough and George[386] separated *cis*- and *trans*-isomers of olefins after their stereospecific epoxidation with *m*-chloroperbenzoic acid. The oxirane derivatives of the *cis*- and *trans*-isomers have different retention times and can be well separated. Hasty[255] described the GC determination of iodine by its reaction with acetone–sulphuric acid (1:1 molar proportions), extraction of the reaction mixture with hexane and injection of the extract into the gas chromatograph. The iodoacetate obtained is sufficiently stable and can be well separated and detected.

4. ABBREVIATIONS USED

BSA	N,O-Bistrimethylsilylacetamide
BSTFA	N,O-Bistrimethylsilyltrifluoroacetamide
DMS	Dimethylsilyl (derivative)
DNPH	Dinitrophenylhydrazone
ECD	Electron-capture detector
FID	Flame-ionization detector

GC	Gas chromatography
HFB	Heptafluorobutyrate
HMDS	Hexamethyldisilazane
HMDSO	Hexamethyldisiloxane
PFP	Pentafluoropropionate
TFA	Trifluoroacetate
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl (derivative)
TMSDEA	Trimethylsilyldiethylamine
TMSIM	Trimethylsilylimidazole

REFERENCES

1 R. G. Ackman, S. N. Hooper, M. Kates, A. K. Sen Gupta, G. Eglinton and I. MacLean, *J. Chromatogr.*, 44 (1969) 256.
2 H. Adlercreutz, S. Laiho and T. Luukkainen, in F. Polvani, G. Surace and G. Luisi (Editors), *Gas Chromatographic Determination of Hormonal Steroids*, Academic Press, New York, London, 1967, p. 69.
3 H. Adlercreutz and T. Luukkainen, in M. B. Lipsett (Editor), *Gas Chromatography of Steroids in Biological Fluids*, Plenum Press, New York, 1965, p. 215.
4 P. Albersheim, D. J. Nevins, P. D. English and A. Karr, *Carbohyd. Res.*, 5 (1967) 340.
5 D. K. Albert, *Anal. Chem.*, 36 (1964) 2034.
6 P. W. Albro and L. Fishbein, *J. Chromatogr.*, 44 (1969) 443.
7 P. W. Albro and L. Fishbein, *J. Chromatogr.*, 55 (1971) 297.
8 N. M. Alexander and R. Scheig, *Anal. Biochem.*, 22 (1968) 187.
9 G. R. Allen and M. J. Saxby, *J. Chromatogr.*, 37 (1968) 312.
10 S. H. Al-Shakir, *Ph.D. Thesis*, University of Georgia, Athens, Ga., 1967; *Diss. Abstr.*, B28 (1968) 2892.
11 D. Anderle and P. Kováč, *J. Chromatogr.*, 49 (1970) 419.
12 D. Anderle, P. Kováč and H. Anderlová, *J. Chromatogr.*, 64 (1972) 368.
13 D. Anderle, M. Petriková and P. Kováč, *J. Chromatogr.*, 58 (1971) 209.
14 M. W. Anders and M. J. Cooper, *Anal. Chem.*, 43 (1971) 1093.
15 M. W. Anders and G. J. Mannering, *Anal. Chem.*, 34 (1962) 730.
16 I. Andersson, B. Norkrans and G. Odham, *Anal. Biochem.*, 53 (1973) 629.
17 E. Änggard and G. Fedvall, *Anal. Chem.*, 41 (1969) 1250.
18 R. G. Annett and P. K. Stumpf, *Anal. Biochem.*, 47 (1972) 638.
19 G. M. Anthony, C. J. W. Brooks, I. MacLean and I. Sangster, *J. Chromatogr. Sci.*, 7 (1969) 623.
20 A. Anton, *Anal. Chem.*, 40 (1968) 1116.
21 T. Aoyama and S. Iguchi, *J. Chromatogr.*, 43 (1969) 253.
22 A. J. Appleby and J. E. O. Mayne, *J. Gas Chromatogr.*, 5 (1967) 266.
23 R. J. Argauer, *Anal. Chem.*, 40 (1968) 122.
24 E. R. Atkinson and J. I. Calouche, *Anal. Chem.*, 43 (1971) 460.
25 J. Attal and S. M. Hendeles, *Anal. Biochem.*, 20 (1967) 394.
26 J. E. Attrill, W. C. Butts, W. T. Rainey and J. W. Holleman, *Anal. Lett.*, 3 (1970) 59.
27 W. A. Aue, C. R. Hastings, K. O. Gerhardt, J. O. Pierce, II, H. H. Hill and R. F. Moseman, *J. Chromatogr.*, 72 (1972) 259.
28 C. A. Bache, L. E. S. John and D. J. Lisk, *Anal. Chem.*, 40 (1968) 1241.
29 E. T. Backer and V. J. Pileggi, *J. Chromatogr.*, 36 (1968) 351.
30 E. Bailey, *Steroids*, 10 (1967) 527.
31 J. J. Bailey, *Anal. Chem.*, 39 (1967) 1485.
32 T. A. Baillie, C. J. W. Brooks and E. C. Horning, *Anal. Lett.*, 5 (1972) 351.
33 T. A. Baillie, C. J. W. Brooks and B. S. Middleditch, *Anal. Chem.*, 44 (1972) 30.
34 C. V. Banks and R. F. Sieck, *Anal. Chem.*, 44 (1972) 2307.
35 R. S. Barratt, R. Belcher, W. I. Stephen and P. C. Uden, *Anal. Chim. Acta*, 59 (1972) 59.

36 E. J. Barron and L. A. Mooney, *Anal. Chem.*, 40 (1968) 1742.
37 R. Bassette, C. R. Brewington and D. P. Schwartz, *Microchem. J.*, 13 (1968) 297.
38 E. Bayer, H. P. Mueller and R. Sievers, *Anal. Chem.*, 43 (1971) 2012.
39 E. Bayer, K. H. Reuthe and F. Born, *Angew. Chem.*, 69 (1957) 640.
40 A. H. Beckett and B. Testa, *J. Chromatogr.*, 69 (1972) 285.
41 R. Belcher, C. R. Jenkins, W. I. Stephen and P. C. Uden, *Talanta*, 17 (1970) 455.
42 R. Belcher, R. J. Martin, W. I. Stephen, D. E. Henderson, D. A. Kamaliza and P. C. Uden, *Anal. Chem.*, 45 (1973) 1197.
43 R. Belcher, M. Pravica, W. I. Stephen and P. C. Uden, *Chem. Commun.*, (1971) 41.
44 R. Belcher, W. I. Stephen, I. J. Thomson and P. C. Uden, *Chem. Commun.*, (1970) 1019.
45 S. Benocci, P. Tarli and P. Neri, *J. Chromatogr.*, 69 (1972) 311.
46 K. Bergström, J. Güntler and R. Blomstrand, *Anal. Chem.*, 34 (1970) 74.
47 C. R. Berret and C. McNeil, *Clin. Chem.*, 12 (1966) 399.
48 F. Berthou, L. Bardou and H. H. Floch, *J. Chromatogr.*, 93 (1974) 149.
49 J. Bezard and M. Bugant, *J. Chromatogr. Sci.*, 7 (1969) 639.
50 P. Biandrate, G. Tognoni, G. Belvedere, A. Frigerio, M. Rizzo and P.L. Morselli, *J. Chromatogr.* 74 (1972) 31.
51 R. Birkofer and A. Ritter, *Chem. Ber.*, 93 (1960) 424.
52 C. T. Bishop and F. P. Cooper, *Can. J. Chem.*, 38 (1960) 388.
53 K. Blau, in H. A. Szymanski (Editor), *Biomedical Applications of Gas Chromatography*, Vol. 2, Plenum Press, New York, 1968, p. 1.
54 K. Blau, in L. R. Mattick and H. A. Szymanski (Editors), *Lectures on Gas Chromatography — 1966*, Plenum Press, New York, 1967, p. 183.
55 K. Blau and A. Darbre, *J. Chromatogr.*, 17 (1965) 445.
56 K. Blau and A. Darbre, *J. Chromatogr.*, 26 (1967) 35.
57 B. Blessington and N. I. Y. Fiagbe, *J. Chromatogr.*, 68 (1972) 259.
58 B. Blessington and N. I. Y. Faigbe, *J. Chromatogr.*, 78 (1973) 343.
59 E. J. Bonelli, *Pesticide Residue Analysis Handbook*, Varian Aerograph, Walnut Creek, Calif., 1966.
60 W. A. Bonner, *J. Chromatogr. Sci.*, 10 (1972) 159.
61 H. B. Boren, P. J. Garegg, L. Kenne, A. Pilotti, S. Svensson and C. G. Swahn, *Acta Chem. Scand.*, 27 (1973) 3557.
62 G. H. Both and W. J. Darby, *Anal. Chem.*, 43 (1971) 831.
63 M. C. Bowman and M. Beroza, *J. Ass. Offic. Anal. Chem.*, 50 (1967) 926.
64 J. W. Boyden and M. Clift, *Z. Anal. Chem.*, 256 (1971) 351.
65 H. Brandenberger and E. Hellbach, *Helv. Chim. Acta*, 50 (1967) 958.
66 B. L. Brian, R. W. Gracy and V. E. Scholes, *J. Chromatogr.*, 63 (1971) 386.
67 B. L. Brian, R. W. Gracy and V. E. Scholes, *J. Chromatogr.*, 66 (1972) 138.
68 T. Briggs and S. R. Lipsky, *Biochim. Biophys. Acta*, 97 (1965) 579.
69 E. Brochmann-Hanssen and T. O. Oke, *J. Pharm. Sci.*, 58 (1969) 370.
70 E. Brochmann-Hanssen and A. B. Svendsen, *J. Pharm. Sci.*, 51 (1962) 1095.
71 C. J. W. Brooks, *Anal. Chem.*, 37 (1965) 636.
72 C. J. W. Brooks, E. Chambaz and E. C. Horning, *Anal. Biochem.*, 19 (1967) 234.
73 C. J. W. Brooks, M. T. Gilbert and J. D. Gilbert, *Anal. Chem.*, 45 (1973) 896.
74 C. J. W. Brooks and D. J. Harvey, *J. Chromatogr.*, 54 (1971) 193.
75 C. J. W. Brooks and I. MacLean, *J. Chromatogr. Sci.*, 9 (1971) 18.
76 C. J. W. Brooks, A. R. Thawley, P. Rocher, B. S. Middleditch, G. M. Anthony and W. G. Stillwell, *J. Chromatogr. Sci.*, 9 (1971) 39.
77 J. B. Brooks, C. C. Alley and R. Jones, *Anal. Chem.*, 44 (1972) 1881.
78 H. Brötell, H. Ehrsson and O. Gyllenhaal, *J. Chromatogr.*, 78 (1973) 293.
79 J. B. Brown, *Biochem. J.*, 60 (1955) 185.
80 A. C. Brownie, H. vander Molen, E. E. Nishizawa and K. B. Eik-Nes, *J. Clin. Endocrinol. Metab.*, 24 (1964) 1091.
81 L. E. Brydia and H. E. Persinger, *Anal. Chem.*, 39 (1967) 1318.
82 L. E. Brydia and F. Willeboordse, *Anal. Chem.*, 40 (1968) 110.
83 H. P. Burchfield, E. E. Storrs, R. J. Wheeler, V. K. Bhat and L. L. Green, *Anal. Chem.*, 45 (1973) 916.

84 A. C. Burgett and J. S. Fritz, *Anal. Chem.*, 44 (1972) 1738.
85 W. C. Butts, *J. Chromatogr. Sci.*, 8 (1970) 474.
86 W. C. Butts, *Anal. Lett.*, 3 (1970) 29.
87 W. C. Butts, *Anal. Biochem.*, 46 (1972) 187.
88 W. C. Butts and C. V. Banks, *Anal. Chem.*, 42 (1970) 133.
89 W. C. Butts and W. T. Rainey, *Anal. Chem.*, 43 (1971) 538.
90 K. A. Caldwell and A. L. Tappel, *J. Chromatogr.*, 32 (1968) 635.
91 P. Capella and E. C. Horning, *Anal. Chem.*, 38 (1966) 316.
92 G. Carlström, *Acta Vet. Scand.*, 9 (1968) 71.
93 W. Carruthers and R. A. W. Johnstone, *Nature (London)*, 185 (1960) 762.
94 W. Carruthers, R. A. W. Johnstone and J. R. Plimmer, *Chem. Ind. (London)*, (1958) 331.
95 J. R. G. Challis and R. B. Heap, *J. Chromatogr.*, 50 (1970) 228.
96 R. A. Chalmers and R. W. E. Watts, *Analyst (London)*, 97 (1972) 951.
97 E. M. Chambaz and E. C. Horning, *Anal. Lett.*, 1 (1967) 201.
98 E. M. Chambaz and E. C. Horning, *Anal. Biochem.*, 30 (1969) 7.
99 J. Chamberlain, B. A. Knights and B. H. Thomas, *J. Endocrinol.*, 28 (1964) 235.
100 J. R. Chapman and E. Bailey, *Anal. Chem.*, 45 (1973) 1636.
101 G. Charransol, F. Bobas-Masson, S. Guillemant and P. Mauvais-Jarvis, *J. Chromatogr.*, 66 (1972) 55.
102 A. S. Y. Chau and W. P. Cochrane, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 1092.
103 A. S. Y. Chau and W. P. Cochrane, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 1220.
104 J. P. Chaytor, B. Crathorne and M. J. Saxby, *J. Chromatogr.*, 70 (1972) 141.
105 Y. M. Choy, G. G. S. Dutton, K. B. Gibney and S. Kabir, *J. Chromatogr.*, 72 (1972) 13.
106 J. Churáček, M. Drahokoupilová, P. Matoušek and K. Komárek, *Chromatographia*, 2 (1969) 493.
107 S. J. Clark and H. H. Wotiz, *Steroids*, 2 (1963) 535.
108 D. D. Clarke, S. Wilk and S. E. Gitlow, *J. Gas Chromatogr.*, 4 (1966) 310.
109 D. D. Clarke, S. Wilk, S. E. Gitlow and M. J. Franklin, in A. Zlatkis (Editor), *Advances in Chromatography 1967*, Chromatography Symposium, Houston, Texas, 1967, p. 145.
110 R. B. Clayton, *Nature (London)*, 190 (1961) 1071.
111 A. J. Cliffe, N. J. Berridge and D. R. Westgarth, *J. Chromatogr.*, 78 (1973) 333.
112 I. C. Cohen, J. Norcup, J. H. A. Ruzicka and B. B. Wheals, *J. Chromatogr.*, 44 (1969) 251.
113 J. R. Coulter and C. S. Hann, *J. Chromatogr.*, 36 (1968) 42.
114 J. H. Copenhaver, *Anal. Biochem.*, 17 (1966) 76.
115 J. A. Corbin and L. B. Rogers, *Anal. Chem.*, 42 (1970) 974.
116 J. A. Corbin and L. B. Rogers, *Anal. Chem.*, 42 (1970) 1786.
117 D. L. Corina, *J. Chromatogr.*, 87 (1973) 254.
118 D. L. Corina and P. M. Dunstan, *Anal. Biochem.*, 53 (1973) 571.
119 R. F. Coward and P. Smith, *J. Chromatogr.*, 45 (1969) 230.
120 P. S. Cowley, F. J. Evans and R. F. A. Ginman, *J. Chromatogr.*, 54 (1971) 185.
121 R. I. Cox and A. R. Bedford, *Steroids*, 3 (1964) 663.
122 B. M. Craig, A. P. Tulloch and N. L. Murty, *J. Amer. Oil Chem. Soc.*, 40 (1963) 61.
123 S. P. Cram and R. S. Juvet, *Anal. Chem.*, 44 (1972) 230.
124 B. Crathorne and M. J. Saxby, *J. Chromatogr.*, 82 (1973) 373.
125 P. A. Cruickshank and J. C. Sheena, *Anal. Chem.*, 36 (1964) 1191.
126 L. M. Cummins and M. J. Fourier, *Anal. Lett.*, 2 (1969) 403.
127 J. C. Dabrowiak and D. W. Cooke, *Anal. Chem.*, 43 (1971) 791.
128 J. M. H. Daemen and W. Dankelman, *J. Chromatogr.*, 78 (1973) 281.
129 C. E. Dalgliesh, E. C. Horning, K. L. Knox and K. Yarger, *Biochem. J.*, 101 (1966) 792.
130 F. C. Dallos and K. G. Koeppl, *J. Chromatogr. Sci.*, 7 (1969) 565.
131 A. Darbre and K. Blau, *Biochim. Biophys. Acta*, 100 (1965) 298.
132 A. Darbre and K. Blau, *J. Chromatogr.*, 17 (1965) 31.
133 A. Darbre and K. Blau, *J. Chromatogr.*, 29 (1967) 49.
134 F. W. Deckert, *J. Chromatogr.*, 64 (1972) 355.
135 F. W. Deckert, *J. Chromatogr.*, 69 (1972) 201.
136 G. A. R. Decroix, J. G. Gobert and R. de Deurwaerder, *Anal. Biochem.*, 25 (1968) 523.
137 L. A. Dee, *Anal. Chem.*, 43 (1971) 1416.
138 P. H. Degen, J. R. DoAmaral and J. D. Barchas, *Anal. Biochem.*, 45 (1972) 634.

139 H. Dekirmenjian and J. W. Maas, *Clin. Chim. Acta*, 32 (1971) 310.
140 P. G. Devaux and E. C. Horning, *Anal. Lett.*, 2 (1969) 637.
141 P. G. Devaux, M. G. Horning, R. M. Hill and E. C. Horning, *Anal. Biochem.*, 41 (1971) 70.
142 P. G. Devaux, M. G. Horning and E. C. Horning, *Anal. Lett.*, 4 (1971) 151.
143 H. G. J. DeWilt, *J. Chromatogr.*, 63 (1971) 379.
144 M. Donike, *J. Chromatogr.*, 42 (1969) 103.
145 M. Donike, *J. Chromatogr.*, 74 (1972) 121.
146 M. Donike, *J. Chromatogr.*, 78 (1973) 273.
147 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 43 (1969) 303.
148 D. Roach and C. W. Gehrke, *J. Chromatogr.*, 44 (1969) 269.
149 D. Roach, C. W. Gehrke and R. W. Zumwalt, *J. Chromatogr.*, 43 (1969) 311.
150 T. P. Dornseifer and J. J. Powers, *J. Food Technol.*, 17 (1963) 118.
151 R. A. Dove, *Anal. Chem.*, 39 (1967) 1188.
152 D. T. Downing, *Anal. Chem.*, 39 (1967) 218.
153 D. T. Downing and R. S. Greene, *Anal. Chem.*, 40 (1968) 827.
154 G. H. Draffan, R. A. Clare and F. M. Williams, *J. Chromatogr.*, 75 (1973) 45.
155 R. J. Dummel and E. Kun, *J. Chromatogr.*, 54 (1971) 130.
156 P. E. Dunagin and J. A. Olson, *Anal. Chem.*, 36 (1964) 756.
157 W. Dünges, *Chromatographia*, 6 (1973) 196.
158 D. E. Durbin and A. Zlatkis, *J. Chromatogr. Sci.*, 8 (1970) 608.
159 G. G. S. Dutton, K. B. Gibney, G. D. Jensen and P. E. Reid, *J. Chromatogr.*, 36 (1968) 152.
160 S. W. Dziedzic, L. M. Bertani, D. D. Clarke and S. E. Gitlow, *Anal. Biochem.*, 47 (1972) 592.
161 C. Eaborn, C. A. Holder, D. M. R. Walton and B. S. Thomas, *J. Chem. Soc., C*, (1969) 2502.
162 C. Eaborn, D. M. R. Walton and B. S. Thomas, *Chem. Ind. (London)*, (1967) 827.
163 D. J. Edwards and K. Blau, *Anal. Biochem.*, 45 (1972) 387.
164 K. B. Eik-Nes and E. C. Horning, *Gas Phase Chromatography of Steroids in Biological Fluids*, Springer, Berlin, Heidelberg, New York, 1968.
165 K. J. Eisentraut, J. D. Griest and R. E. Sievers, *Anal. Chem.*, 43 (1971) 2003.
166 W. H. Elliot, L. B. Walsh, Meimeimui, M. A. Thorne and C. M. Siegfried, *J. Chromatogr.*, 44 (1969) 452.
167 S. Eriksson and J. Sjöquist, *Biochim. Biophys. Acta*, 45 (1960) 290.
168 G. G. Esposito, *Anal. Chem.*, 40 (1968) 1902.
169 G. G. Esposito and H. M. Swann, *Anal. Chem.*, 41 (1969) 1118.
170 W. J. Esselman and C. O. Clagett, *J. Lipid Res.*, 10 (1969) 234.
171 A. Estas and A. Dumont, *J. Chromatogr.*, 82 (1973) 307.
172 F. L. Estes and R. C. Bachmann, *Anal. Chem.*, 38 (1966) 1178.
173 R. T. Evans, *J. Chromatogr.*, 88 (1974) 398.
174 J. Eyem and J. Sjöquist, *Anal. Biochem.*, 52 (1973) 255.
175 H. M. Fales, T. M. Jaouni and J. F. Babashak, *Anal. Chem.*, 45 (1973) 2302.
176 H. M. Fales and T. Luukkainen, *Anal. Chem.*, 37 (1965) 955.
177 E. Fedeli and M. Cirimele, *J. Chromatogr.*, 15 (1964) 435.
178 E. Felder, U. Tiepolo and A. Mengassini, *J. Chromatogr.*, 82 (1973) 291.
179 E. Felder, U. Tiepolo and A. Mengassini, *J. Chromatogr.*, 82 (1973) 390.
180 D. G. Ferry and E. G. McQueen, *J. Chromatogr.*, 76 (1973) 233.
181 R. J. Fessenden and D. F. Crowe, *J. Org. Chem.*, 26 (1961) 4638.
182 J. A. Fioriti, M. J. Kanuk and R. J. Sims, *J. Chromatogr. Sci.*, 7 (1969) 448.
183 A. L. Fisher, A. M. Parfitt and H. M. Lloyd, *J. Chromatogr.*, 65 (1972) 493.
184 G. T. Flint and W. A. Aue, *J. Chromatogr.*, 52 (1970) 487.
185 R. Fontaine, B. Santoni, C. Pommier and G. Guiochon, *Chromatographia*, 3 (1970) 532.
186 J. K. Foreman, T. A. Gough and E. A. Walker, *Analyst (London)*, 95 (1970) 797.
187 B. Fournet and J. Montreuil, *J. Chromatogr.*, 75 (1973) 29.
188 B. H. Freeman, A. M. Stephen and P. van der Bijl, *J. Chromatogr.*, 73 (1972) 29.
189 J. M. Frére and W. G. Verly, *J. Chromatogr.*, 49 (1970) 425.
190 S. Friedman, C. Zahn, M. Kaufman and I. Wender, *Bur. Mines Bull.*, (1963) 609.
191 S. Friedman and M. L. Kaufman, *Anal. Chem.*, 38 (1966) 144.
192 A. Frigerio, P. Martelli and R. M. Baker, *J. Chromatogr.*, 81 (1973) 139.
193 S.-C. J. Fu, D. S. H. Mak, *J. Chromatogr.*, 54 (1971) 205.

194 S.-C. J. Fu and D. S. H. Mak, *J. Chromatogr.*, 78 (1973) 211.
195 T. Furuya, *J. Chromatogr.*, 19 (1965) 607.
196 T. Furuya, *J. Chromatogr.*, 18 (1965) 152.
197 T. Furuya, S. Shibata and H. Iizuka, *J. Chromatogr.*, 21 (1966) 116.
198 D. F. Gadbois, J. M. Mendelsohn and L. J. Ronsivalli, *Anal. Chem.*, 37 (1965) 1776.
199 D. F. Gadbois, P. G. Schreurer and F. J. King, *Anal. Chem.*, 40 (1968) 1362.
200 A. Gazzaniga and E. Pezzotti, *J. Chromatogr.*, 81 (1973) 71.
201 C. W. Gehrke, D. Roach, R. W. Zumwalt, D. L. Stalling and L. L. Wall, *Quantitative Gas Chromatography of Amino Acids in Proteins and Biological Substances*, Analytical Biochemistry Laboratories Inc., Columbia, 1968.
202 C. W. Gehrke, K. Kuo and R. W. Zumwalt, *J. Chromatogr.*, 57 (1971) 209.
203 C. W. Gehrke and D. B. Lakings, *J. Chromatogr.*, 61 (1971) 45.
204 C. W. Gehrke and K. Leimer, *J. Chromatogr.*, 53 (1970) 201.
205 C. W. Gehrke and K. Leimer, *J. Chromatogr.*, 57 (1971) 219.
206 C. W. Gehrke, H. Nakamoto and R. W. Zumwalt, *J. Chromatogr.*, 45 (1969) 24.
207 C. W. Gehrke and F. Sharokhi, *Anal. Biochem.*, 15 (1966) 97.
208 C. W. Gehrke and D. L. Stalling, *Separ. Sci.*, 3 (1967) 101.
209 C. W. Gehrke, D. L. Stalling and C. D. Ruyle, *Biochem. Biophys. Res. Commun.*, 28 (1967) 869.
210 C. W. Gehrke and H. Takeda, *J. Chromatogr.*, 76 (1973) 63.
211 C. W. Gehrke, R. W. Zumwalt and K. Kuo, *J. Chromatogr.*, 57 (1971) 193.
212 C. W. Gehrke, R. W. Zumwalt and L. L. Wall, *J. Chromatogr.*, 37 (1968) 398.
213 T. Gejvall, *J. Chromatogr.*, 90 (1974) 157.
214 C. Genty, C. Houin, P. Malherbe and R. Schott, *Anal. Chem.*, 43 (1971) 235.
215 K. O. Gerhardt and W. A. Aue, *J. Chromatogr.*, 82 (1973) 382.
216 E. Gil-Av, R. Charles and G. Fischer, *J. Chromatogr.*, 17 (1965) 408.
217 H. H. Gill, *Anal. Chem.*, 36 (1964) 1201.
218 C. R. Glowacki, P. J. Menardi and W. E. Link, *J. Amer. Oil Chem. Soc.*, 47 (1970) 225.
219 D. B. Gower and B. S. Thomas, *J. Chromatogr.*, 36 (1968) 338.
220 O. Grahl-Nielsen and E. Solheim, *J. Chromatogr.*, 69 (1972) 366.
221 G. M. Gray, *J. Chromatogr.*, 4 (1960) 52.
222 G. M. Gray, *J. Chromatogr.*, 6 (1961) 236.
223 R. H. Greeley, *J. Chromatogr.*, 88 (1974) 229.
224 R. Greenhalgh and P. J. Wood, *J. Chromatogr.*, 82 (1973) 410.
225 N. L. Gregory, *J. Chromatogr.*, 36 (1968) 342.
226 L. J. Griggs, A. Post, E. R. White, J. A. Finkelstein, W. E. Moeckel, K. G. Holden, J. E. Zarembo and J. A. Weisbach, *Anal. Biochem.*, 43 (1971) 369.
227 C. Grunwald and R. G. Lockard, *J. Chromatogr.*, 52 (1970) 491.
228 S. Gunner, J. K. N. Jones and M. B. Perry, *Chem. Ind. (London)*, (1961) 255.
229 D. Gupta, E. Breitmaier, G. Jung, G. V. Lucadou, H. Pauschmann and W. Voelter, *Chromatographia*, 4 (1971) 572.
230 K. G. Gupta and P. J. Scheurer, *Steroids*, 13 (1969) 352.
231 P. B. Hagen and W. Black, *Can. J. Biochem.*, 43 (1965) 309.
232 J. K. Haken, *J. Gas Chromatogr.*, 1 (1963) 30.
233 J. K. Haken and V. Khemangkorn, *J. Chromatogr. Sci.*, 10 (1972) 41.
234 B. Halpern, V. A. Close, A. Wegmann and W. J. Westley, *Tetrahedron Lett.*, 27 (1968) 3119.
235 H. Halvarson, *J. Chromatogr.*, 57 (1971) 406.
236 H. Halvarson, *J. Chromatogr.*, 66 (1972) 35.
237 G. M. Hamberg, W. G. Niehaus and B. Samuelsson, *Anal. Biochem.*, 22 (1968) 145.
238 K. Hammarstrand and E. J. Bonelli, *Derivative Formation in Gas Chromatography*, Varian Aerograph, Walnut Creek, Calif., 1968.
239 S. Hammarström and M. Hamberg, *Anal. Biochem.*, 52 (1973) 169.
240 R. L. Hancock, *J. Gas Chromatogr.*, 4 (1966) 363.
241 R. L. Hancock, *J. Gas Chromatogr.*, 6 (1968) 431.
242 R. L. Hancock, *J. Chromatogr. Sci.*, 7 (1969) 366.
243 R. L. Hancock and D. L. Coleman, *Anal. Biochem.*, 10 (1965) 365.
244 I. Hanin and D. J. Jenden, *Biochem. Pharmacol.*, 18 (1969) 837.
245 L. C. Hansen, W. G. Scribner, T. W. Gilbert and R. E. Sievers, *Anal. Chem.*, 43 (1971) 349.

246 C. J. Hardy, *J. Chromatogr.*, 13 (1964) 372.
247 J. P. Hardy and S. L. Kerrin, *Anal. Chem.*, 44 (1972) 1497.
248 R. E. Harman, J. L. Patterson and W. J. E. VandenHeuvel, *Anal. Biochem.*, 25 (1968) 452.
249 D. J. Harvey and M. G. Horning, *J. Chromatogr.*, 76 (1973) 51.
250 D. J. Harvey and M. G. Horning, *J. Chromatogr.*, 79 (1973) 65.
251 T. Hashizume and Y. Sasaki, *Anal. Biochem.*, 15 (1966) 199.
252 T. Hashizume and Y. Sasaki, *Anal. Biochem.*, 15 (1966) 364.
253 T. Hashizume and Y. Sasaki, *Anal. Biochem.*, 21 (1967) 316.
254 T. Hashizume and Y. Sasaki, *Anal. Biochem.*, 24 (1968) 232.
255 R. H. Hasty, *Microchim. Acta*, (1971) 348.
256 E. Hautala and M. L. Weaver, *Anal. Biochem.*, 30 (1969) 32.
257 R. A. Heacock and J. E. Forrest, *J. Chromatogr.*, 81 (1973) 57.
258 E. J. Hedgley, O. Meresz, W. G. Overend and R. Rennie, *Chem. Ind. (London)*, (1960) 938.
259 R. W. Hemingway, W. E. Hillis and N. K. Bruerton, *J. Chromatogr.*, 50 (1970) 391.
260 S. F. Herb, *J. Amer. Oil Chem. Soc.*, 45 (1968) 784.
261 J. E. Herz and E. González, *J. Chromatogr.*, 34 (1968) 251.
262 D. M. Hilker and J. M. L. Mee, *J. Chromatogr.*, 76 (1973) 239.
263 J. B. Himes and I. J. Dowbak, *J. Gas Chromatogr.*, 3 (1965) 194.
264 K. Hirano, K. Mori, S. Kawai and T. Ohno, *J. Chromatogr.*, 64 (1972) 174.
265 M. Hiroi and S. Kushinski, *Microchim. Acta*, (1969) 1160.
266 C. Hishta, D. L. Mays and M. Garofalo, *Anal. Chem.*, 43 (1971) 1530.
267 N. E. Hoffman and T. A. Killinger, *Anal. Chem.*, 41 (1969) 162.
268 E. R. Holden, W. M. Jones and M. Beroza, *J. Agr. Food Chem.*, 17 (1969) 56.
269 R. H. Holm, G. W. Everett and A. Chakravorty, *Progr. Inorg. Chem.*, 7 (1966) 83.
270 B. Holmstedt, W. J. A. VandenHeuvel, W. R. Gardiner and E. C. Horning, *Anal. Biochem.*, 8 (1964) 151.
271 Z. Horii, M. Makita and Y. Tamura, *Chem. Ind. (London)*, (1965) 1494.
272 E. C. Horning and M. G. Horning, in A. Zlatkis (Editor), *Advances in Chromatography 1970*, Chromatography Symposium, Houston, Texas, 1970, p. 226.
273 E. C. Horning, M. G. Horning, N. Ikekawa, E. M. Chambaz and P. I. Jaakonmäki, *J. Gas Chromatogr.*, 5 (1967) 283.
274 E. C. Horning and B. F. Maume, *J. Chromatogr. Sci.*, 7 (1969) 411.
275 M. G. Horning, E. A. Boucher and A. M. Moss, *J. Gas Chromatogr.*, 5 (1967) 297.
276 M. G. Horning, E. A. Boucher, A. M. Moss and E. C. Horning, *Anal. Lett.*, 1 (1968) 713.
277 M. G. Horning, G. Gasparrini and E. C. Horning, *J. Chromatogr. Sci.*, 7 (1969) 267.
278 M. G. Horning, K. L. Knox, C. E. Dalgliesh and E. C. Horning, *Anal. Biochem.*, 17 (1966) 244.
279 M. G. Horning, A. M. Moss, E. A. Boucher and E. C. Horning, *Anal. Lett.*, 1 (1968) 311.
280 M. G. Horning, A. M. Moss and E. C. Horning, *Anal. Biochem.*, 22 (1968) 284.
281 M. G. Horning, A. M. Moss and E. C. Horning, *Biochim. Biophys. Acta*, 148 (1967) 597.
282 I. Hornstein, J. A. Alford, L. E. Elliott and P. F. Crowe, *Anal. Chem.*, 32 (1960) 540.
283 H. B. Hucker and J. K. Miller, *J. Chromatogr.*, 32 (1968) 408.
284 N. Ikekawa, O. Hoshino, R. Watanuki, H. Orimo and T. Fujita, *Anal. Biochem.*, 17 (1966) 16.
285 P. H. B. Ingle, H. Y. Koh and R. H. Perrett, *J. Chromatogr.*, 81 (1973) 79.
286 T. Imanari, Y. Arakawa and Z. Tamura, *Chem. Pharm. Bull.*, 17 (1969) 1967.
287 A. Islam and A. Darbre, *J. Chromatogr.*, 43 (1969) 11.
288 P. I. Jaakonmäki and J. E. Stouffer, *J. Gas Chromatogr.*, 5 (1967) 303.
289 D. B. Jack and W. Riess, *J. Chromatogr.*, 88 (1974) 173.
290 M. Jacobson, J. F. O'Brien and C. Hegcoth, *Anal. Biochem.*, 25 (1968) 363.
291 E. F. Jansen and N. C. Baglan, *J. Chromatogr.*, 38 (1968) 18.
292 G. Janssen and H. Vanderhaeghe, *J. Chromatogr.*, 82 (1973) 297.
293 R. W. Jelliffe and D. H. Blankenhorn, *J. Chromatogr.*, 12 (1963) 268.
294 E. Jellum, V. A. Bacon and W. Patton, *Anal. Biochem.*, 31 (1969) 339.
295 D. J. Jenden, B. Campbell and M. Roch, *Anal. Biochem.*, 35 (1970) 209.
296 D. J. Jenden, I. Hanin and S. I. Lamb, *Anal. Chem.*, 40 (1968) 125.
297 D. C. Johnson and E. G. Hammond, *J. Amer. Oil Chem. Soc.*, 48 (1971) 653.
298 E. P. Jones and V. L. Davison, *J. Amer. Oil Chem. Soc.*, 42 (1965) 121.
299 H. G. Jones, J. K. N. Jones and M. B. Perry, *Can. J. Chem.*, 40 (1962) 1559.

300 L. A. Jones and R. J. Monroe, *Anal. Chem.*, 37 (1965) 935.
301 P. Jones and G. Nickless, *J. Chromatogr.*, 76 (1973) 285.
302 P. Jones and G. Nickless, *J. Chromatogr.*. 89 (1974) 201.
303 T. C. Jones and I. Schmeltz, *Tob. Sci.*, 166 (1968) 20.
304 J. Jönsson, J. Eyem and J. Sjöquist, *Anal. Biochem.*, 51 (1973) 204.
305 G. H. Jouvenaz, D. H. Nugteren, R. K. Beerthuis and D. A. Vaaldorp, *Biochim. Biophys. Acta*, 202 (1970) 231.
306 S. R. Juvet and R. L. Fisher, *Anal. Chem.*, 38 (1966) 1860.
307 J. Kagan and T. J. Mabry, *Anal. Chem.*, 37 (1965) 288.
308 Z. Kahane, J. H. Mowat and P. Vestergaard, *Clin. Chim. Acta*, 26 (1969) 307.
309 H. Kallio and R. R. Linko, *J. Chromatogr.*, 76 (1973) 229.
310 H. Kallio, R. Linko and J. Kaitaranta, *J. Chromatogr.*, 65 (1972) 355.
311 R. Kannan, A. Rajiah, M. Subbaram and K. T. Achaya, *J. Chromatogr.*, 55 (1971) 402.
312 A. Karmen, *J. Lipid Res.*, 8 (1967) 234.
313 F. Karoum, C. O. Anah, C. R. J. Ruthven and M. Sandler, *Clin. Chim. Acta*, 24 (1969) 341.
314 F. Karoum, F. Cattabeni, E. Costa, C. R. J. Ruthven and M. Sandler, *Anal. Biochem.*, 47 (1972) 550.
315 T. Katagi, A. Horii, Y. Oomura, H. Miyakawa, T. Kyu, Y. Ikeda and K. Isoi, *J. Chromatogr.*, 79 (1973) 45.
316 H. Kato, K. Yamada, S. Kawai and T. Ohno, *J. Chromatogr.*, 82 (1973) 323.
317 M. L. Kaufman, S. Friedman and I. Wender, *Anal. Chem.*, 39 (1967) 1011.
318 F. K. Kawahara, *Anal. Chem.*, 40 (1968) 1009.
319 F. K. Kawahara, *Anal. Chem.*, 40 (1968) 2073.
320 S. Kawai and Z. Tamura, *Chem. Pharm. Bull.*, 15 (1967) 1493.
321 R. W. Kelly, *J. Chromatogr.*, 43 (1969) 229.
322 R. W. Kelly, *Tetrahedron Lett.*, (1969) 967.
323 H. Kern, P. Schilling and S. H. Müller, *Gas Chromatographic Analysis of Pharmaceuticals and Drugs*, Varian Aerograph, Walnut Creek, Calif., 1968.
324 S. M. Kim, R. Bentley and C. C. Sweeley, *Carbohyd. Res.*, 5 (1967) 373.
325 H. W. Kircher, *Anal. Chem.*, 32 (1960) 1103.
326 J. J. Kirkland, *Anal. Chem.*, 32 (1960) 1388.
327 M. A. Kirschner and H. M. Fales, *Anal. Chem.*, 34 (1962) 1548.
328 M. A. Kirschner and J. P. Taylor, *Anal. Biochem.*, 30 (1969) 346.
329 A. Kito, Y. Miyake, H. Kobayashi and K. Ueno, *Bunseki Kagaku (Jap. Anal.)*, 20 (1971) 1363.
330 G. W. Kittinger, *Steroids*, 3 (1964) 21.
331 J. F. Klebe, H. Finkbeiner and D. M. White, *J. Amer. Chem. Soc.*, 88 (1966) 3390.
332 H. P. Klemm, U. Hintze and G. Gercken, *J. Chromatogr.*, 75 (1973) 19.
333 B. A. Knights, *J. Gas Chromatogr.*, 5 (1967) 273.
334 B. A. Knights and G. H. Thomas, *Nature (London)*, 194 (1962) 833.
335 H. Ko and M. E. Royer, *J. Chromatogr.*, 88 (1974) 253.
336 W. Korytnyk, in L. R. Mattick and H. A. Szymanski (Editors), *Lectures on Gas Chromatography —1966*, Plenum Press, New York, 1967, p. 89.
337 W. Korytnyk, G. Fricke and B. Paul, *Anal. Biochem.*, 17 (1966) 66.
338 M. Kowblansky, B. M. Scheinthal, G. D. Cravello and L. Chavetz, *J. Chromatogr.*, 76 (1973) 467.
339 N. Lachovitzki and B. Björklund, *Anal. Biochem.*, 38 (1970) 446.
340 D. B. Lakings and C. W. Gehrke, *J. Chromatogr.*, 62 (1971) 347.
341 M. A. Lambert and C. W. Moss, *J. Chromatogr.*, 74 (1972) 335.
342 W. M. Lamkin and C. W. Gehrke, *Anal. Chem.*, 37 (1965) 383.
343 W. M. Lamkin, N. S. Jones, T. Pan and D. N. Ward, *Anal. Biochem.*, 58 (1974) 549.
344 W. M. Lamkin, J. W. Weatherford, N. S. Jones, T. Pan and D. N. Ward, *Anal. Biochem.*, 58 (1974) 422.
345 D. G. Lance and J. K. N. Jones, *Can. J. Chem.*, 45 (1967) 1995.
346 R. A. Landowne and S. R. Lipsky, *Anal. Chem.*, 35 (1963) 532.
347 R. A. Landowne and S. R. Lipsky, *Nature (London)*, 199 (1963) 141.
348 S. H. Langer, S. Connell and I. Wender, *J. Org. Chem.*, 23 (1958) 50.
349 S. H. Langer and P. Pantages, *Nature (London)*, 191 (1961) 141.
350 S. H. Langer, P. Pantages and I. Wender, *Chem. Ind. (London)*, (1958) 1664.
351 T. W. Larkham and J. S. Pagington, *J. Chromatogr.*, 28 (1967) 422.

352 P. A. Larson, G. R. Honold and W. G. Hobbs, *J. Chromatogr.*, 90 (1974) 345.
353 J. L. Laseter, J. D. Weete, A. Albert and C. H. Walkinshaw, *Anal. Lett.*, 4 (1971) 671.
354 W. F. Lehnhardt and R. J. Winzler, *J. Chromatogr.*, 34 (1968) 471.
355 J. Lehrfeld, *J. Chromatogr., Sci.* 9 (1971) 757.
356 K. C. Leibman and E. Ortiz, *J. Chromatogr.*, 32 (1968) 757.
357 W. J. Lewicki and J. R. Edwards, *Anal. Lett.*, 3 (1970) 151.
358 Liat Tan, *J. Chromatogr.*, 45 (1969) 68.
359 C. Litchfield, M. Farquer and R. Reiser, *J. Amer. Oil Chem. Soc.*, 41 (1964) 588.
360 M. H. Litchfield and T. Green, *Analyst (London)*, 95 (1970) 168.
361 J. B. F. Lloyd and B. R. G. Roberts, *J. Chromatogr.*, 77 (1973) 228.
362 T. Lloyd, S. Markey and N. Weiner, *Anal. Biochem.*, 42 (1971) 108.
363 L. J. Lohr and R. W. Warren, *J. Chromatogr.*, 8 (1962) 127.
364 J. E. Lovelock, *Nature (London)*, 189 (1961) 729.
365 J. A. Lubkowitz, *J. Chromatogr.*, 63 (1971) 370.
366 T. Luukkainen and H. Adlercreutz, *Biochim. Biophys. Acta*, 70 (1963) 700.
367 T. Luukkainen, W. J. A. VandenHeuvel, E. O. A. Haahti and E. C. Horning, *Biochim. Biophys. Acta*, 52 (1961) 599.
368 J. MacGee, *Anal. Biochem.*, 14 (1966) 305.
369 J. MacGee and K. G. Allen, *Anal. Biochem.*, 42 (1970) 1672.
370 S. Makisumi and H. A. Saroff, *J. Gas Chromatogr.*, 3 (1965) 21.
371 M. Margosis, *J. Chromatogr.*, 47 (1970) 341.
372 D. M. Marmion, R. G. White, L. H. Bille and K. H. Ferber, *J. Gas Chromatogr.*, 4 (1966) 190.
373 G. E. Martin and J. S. Swinehardt, *Anal. Chem.*, 38 (1966) 1789.
374 H. F. Martin and J. L. Driscoll, *Anal. Chem.*, 38 (1966) 345.
375 Y. Maruyama and A. E. Takemori, *Anal. Biochem.*, 49 (1972) 240.
376 M. E. Mason, M. E. Eager and G. R. Waller, *Anal. Chem.*, 36 (1964) 587.
377 M. E. Mason, B. Johnson and M. C. Hamming, *Anal. Chem.*, 37 (1965) 760.
378 M. E. Mason and G. R. Waller, *Anal. Chem.*, 36 (1964) 583.
379 P. S. Mason and E. D. Smith, *J. Gas Chromatogr.*, 4 (1966) 398.
380 D. R. Mattheuis, W. D. Shults, M. R. Guerin and J. A. Dean, *Anal. Chem.*, 43 (1971) 1582.
381 B. Maume, W. E. Wilson and E. C. Horning, *Anal. Lett.*, 1 (1968) 401.
382 W. J. McBride and J. D. Klingman, in L. R. Mattick and H. A. Szymanski (Editors), *Lectures on Gas Chromatography —1966*, Plenum Press, New York, 1967, p. 25.
383 W. J. McBride and J. D. Klingman, *Anal. Biochem.*, 25 (1968) 109.
384 N. K. McCallum and R. J. Armstrong, *J. Chromatogr.*, 78 (1973) 303.
385 M. A. McClure, *J. Chromatogr.*, 54 (1971) 25.
386 L. M. McDonough and D. A. George, *J. Chromatogr. Sci.*, 8 (1970) 158.
387 W. A. McGugan and S. G. Howsam, *J. Chromatogr.*, 82 (1973) 370.
388 A. G. McInnes, D. H. Ball, F. P. Cooper and C. T. Bishop, *J. Chromatogr.*, 1 (1958) 556.
389 C. E. McCone and R. J. Hance, *J. Chromatogr.*, 69 (1972) 204.
390 J. M. L. Mee and R. W. Stanley, *J. Chromatogr.*, 76 (1973) 242.
391 E. Menini, *Biochem. J.*, 94 (1965) 15 P.
392 I. Merits, *J. Lipid Res.*, 3 (1962) 126.
393 L. D. Metcalfe and R. J. Martin, *Anal. Chem.*, 44 (1972) 403.
394 L. D. Metcalfe and A. A. Schmitz, *Anal. Chem.*, 33 (1961) 363.
395 L. D. Metcalfe, A. A. Schmitz and J. R. Pelka, *Anal. Chem.*, 38 (1966) 514.
396 H. T. Miles and H. M. Fales, *Anal. Chem.*, 34 (1962) 860.
397 V. Miller and V. Pacáková, *Chem. Listy*, 67 (1973) 1121.
398 P. W. D. Mitchell, *J. Chromatogr.*, 76 (1973) 236.
399 O. Mlejnek, *J. Chromatogr.*, 70 (1972) 59.
400 A. C. Moffat and E. C. Horning, *Anal. Lett.*, 3 (1970) 205.
401 A. C. Moffat, E. C. Horning, S. B. Matin and M. Rowland, *J. Chromatogr.*, 66 (1972) 255.
402 E. D. Morgan and C. F. Poole, *J. Chromatogr.*, 89 (1974) 225.
403 S. Mori, M. Furusawa and T. Takeuchi, *Anal. Chem.*, 42 (1970) 138.
404 S. Mori, M. Furusawa and T. Takeuchi, *Anal. Chem.*, 42 (1970) 959.
405 S. Mori, M. Furusawa and T. Takeuchi, *J. Chromatogr. Sci.*, 8 (1970) 477.
406 Y. Mori and T. Sato, *J. Chromatogr.*, 76 (1973) 133.

407 G. P. Morie and T. R. Sweet, *Anal. Chem.*, 37 (1965) 1553.
408 H. Morita, *J. Chromatogr.*, 71 (1972) 149.
409 I. M. Morrison and M. B. Perry, *Can. J. Biochem.*, 44 (1966) 1115.
410 W. R. Morrison and L. M. Smith, *J. Lipid Res.*, 5 (1964) 600.
411 R. A. Morrissette and W. E. Link, *J. Gas Chromatogr.*, 3 (1965) 67.
412 R. W. Moshier and R. E. Sievers, *Gas Chromatography of Metal Chelates*, Pergamon Press, New York, 1965.
413 C. W. Moss and M. A. Lambert, *J. Chromatogr.*, 60 (1971) 134.
414 E. H. Mougey, D. R. Collins, R. M. Rose and J. W. Mason, *Anal. Biochem.*, 27 (1966) 354.
415 S. J. Mulé, *Anal. Chem.*, 36 (1964) 1907.
416 E. Mussini and F. Marcucci, *J. Chromatogr.*, 26 (1967) 481.
417 T. Nagai, S. Hashimoto, I. Yamane and A. Mori, *J. Amer. Oil Chem. Soc.*, 47 (1970) 505.
418 K. Nakagawa, N. L. McNiven, E. Forchielli, A. Vermeulen and R. I. Dorfman, *Steroids*, 7 (1966) 329.
419 T. Nambara and Y. H. Bae, *J. Chromatogr.*, 64 (1972) 239.
420 N. Narasimhachari and P. Vouros, *Anal. Biochem.*, 45 (1972) 154.
421 N. Narasimhachari and P. Vouros, *J. Chromatogr.*, 70 (1972) 135.
422 M. Naruse, K. Hirano, S. Kawai, T. Ohno and Y. Masada, *J. Chromatogr.*, 82 (1973) 331.
423 G. Neurath and W. Lüttich, *J. Chromatogr.*, 34 (1968) 253.
424 G. Neurath and W. Lüttich, *J. Chromatogr.*, 34 (1968) 257.
425 J. G. Nikelly, *Anal. Chem.*, 36 (1964) 2244.
426 T. Noguchi, H. Kaseda, N. Konishi and R. Kido, *J. Chromatogr.*, 55 (1971) 291.
427 H. E. Nordby and S. Nagy, *J. Chromatogr.*, 75 (1973) 187.
428 M. Novotný and J. Janák, *Chem. Listy*, 66 (1972) 693.
429 M. Novotný and A. Zlatkis, *J. Chromatogr. Sci.*, 8 (1970) 346.
430 T. Okuda and K. Konishi, *Chem. Commun.*, (1969) 796.
431 F. I. Onuska and W. R. Boos, *Anal. Chem.*, 45 (1973) 967.
432 R. Osiewicz, V. Aggarwal, R. H. Young and I. Sunshine, *J. Chromatogr.*, 88 (1974) 157.
433 C. Pace-Asciak and L. S. Wolfe, *J. Chromatogr.*, 56 (1971) 129.
434 K. D. Parker, J. A. Wright, A. F. Halpern and C. H. Hine, *J. Forensic Sci. Soc.*, 8 (1968) 125.
435 W. Parr and P. Y. Howard, *J. Chromatogr.*, 66 (1972) 141.
436 W. Parr and P. Y. Howard, *J. Chromatogr.*, 67 (1972) 227.
437 W. Parr, J. Pleterski, C. Yang and E. Bayer, *J. Chromatogr. Sci.*, 9 (1971) 141.
438 W. Parr, C. Yang, J. Pleterski and E. Bayer, *J. Chromatogr.*, 50 (1970) 510.
439 J. S. Parsons, *J. Gas Chromatogr.*, 5 (1967) 254.
440 J. S. Parsons, *J. Chromatogr. Sci.*, 11 (1973) 659.
441 E. D. Pellizzari, J. H. Brown, P. Talbot, R. W. Farmer and L. F. Fabre, *J. Chromatogr.*, 55 (1971) 281.
442 W. Pereira, V. A. Bacon, W. Patton, B. Halpern and G. E. Pollock, *Anal. Lett.*, 3 (1970) 23.
443 R. W. Pero, D. Harvan, R. G. Owens and J. P. Snow, *J. Chromatogr.*, 65 (1972) 501.
444 M. B. Perry, *Can. J. Biochem.*, 42 (1964) 451.
445 M. B. Perry, G. A. Adams and D. H. Shaw, *J. Chromatogr.*, 44 (1969) 614.
446 M. B. Perry and R. K. Hulyalkar, *Can. J. Biochem.*, 43 (1965) 573.
447 B. C. Pettitt, D. G. Simonds and A. Zlatkis, *J. Chromatogr. Sci.*, 7 (1969) 645.
448 B. C. Pettitt and J. E. Stouffer, *J. Chromatogr. Sci.*, 8 (1970) 735.
449 A. E. Pierce, *Silylation of Organic Compounds*, Pierce Chemical Co., Rockford, Ill., 1968.
450 H. B. Pionke, G. Chesters and D. E. Armstrong, *Analyst (London)*, 94 (1969) 900.
451 J. J. Pisano and J. Bronzert, *Anal. Biochem.*, 45 (1972) 43.
452 J. J. Pisano, W. J. A. VandenHeuvel and E. C. Horning, *Biochim. Biophys. Res. Commun.*, 7 (1962) 82.
453 R. Poclington, *Anal. Biochem.*, 45 (1972) 403.
454 G. E. Pollock, *Anal. Chem.*, 39 (1967) 1194.
455 G. E. Pollock, V. I. Oyama and R. D. Johnson, *J. Gas Chromatogr.*, 3 (1965) 174.
456 L. D. Quinn and M. E. Hobbs, *Anal. Chem.*, 30 (1958) 1400.
457 B. Radhakrishnamurthy, E. R. Dalferes and G. J. Berenson, *Anal. Biochem.*, 17 (1966) 545.
458 A. Rajiah, M. R. Subbaram and K. T. Achaya, *J. Chromatogr.*, 38 (1968) 35.
459 K. M. Rajkowski and G. D. Broadhead, *J. Chromatogr.*, 69 (1972) 373.

460 J. W. Ralls, *Anal. Chem.*, 32 (1960) 332.
461 J. W. Ralls, *Anal. Chem.*, 36 (1964) 946.
462 M. Rangarajan, R. E. Ardrey and A. Darbre, *J. Chromatogr.*, 87 (1973) 499.
463 F. Raulin and B. N. Khare, *J. Chromatogr.*, 75 (1973) 13.
464 F. Raulin, P. Shapshak and B. N. Khare, *J. Chromatogr.*, 73 (1972) 35.
465 A. H. Richards and W. B. Mason, *Anal. Chem.*, 38 (1966) 1751.
466 E. W. Robb and J. J. Westbrook, III, *Anal. Chem.*, 35 (1963) 1644.
467 G. Roda and A. Zamorani, *J. Chromatogr.*, 46 (1970) 315.
468 M. Rogozinski, *J. Gas Chromatogr.*, 2 (1964) 136.
469 P. Ronkainen and S. Brummer, *J. Chromatogr.*, 28 (1967) 259.
470 P. Ronkainen and S. Brummer, *J. Chromatogr.*, 45 (1969) 341.
471 C.-G. Rosen, T. Gejvall and L.-O. Andersson, *Biochim. Biophys. Acta*, 221 (1970) 207.
472 H. Rosenquist, H. Kallio and V. Nurmikko, *Anal. Biochem.*, 46 (1972) 224.
473 J. Rosmus and Z. Deyl, *J. Chromatogr.*, 70 (1972) 221.
474 R. Ross and T. Shafik, *J. Chromatogr. Sci.*, 11 (1973) 46.
475 W. D. Ross and R. E. Sievers, in A. B. Littlewood (Editor), *Gas Chromatography 1966*, Institute of Petroleum, London, 1967, p. 272.
476 W. D. Ross and R. E. Sievers, *Anal. Chem.*, 41 (1969) 1109.
477 W. D. Ross, R. E. Sievers and G. Wheeler, *Anal. Chem.*, 37 (1965) 598.
478 W. D. Ross and G. Wheeler, *Anal. Chem.*, 36 (1964) 266.
479 K. Rühlmann, *Chem. Ber.*, 94 (1961) 1876.
480 K. Rühlmann and W. Giesecke, *Angew. Chem.*, 73 (1961) 113.
481 N. Sakauchi and E. C. Horning, *Anal. Lett.*, 4 (1971) 41.
482 G. A. Sarfaty and H. M. Fales, *Anal. Chem.*, 42 (1970) 288.
483 Y. Sasaki and T. Hashizume, *Anal. Biochem.*, 16 (1966) 1.
484 A. Sato and E. von Rudloff, *Can. J. Chem.*, 42 (1964) 635.
485 J. Savory, P. Mushak and F. W. Sunderman, *Anal. Chem.*, 42 (1970) 294.
486 J. S. Sawardeker, J. H. Sloneker and A. Jeanes, *Anal. Chem.*, 37 (1965) 1602.
487 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
488 M. Schnitzer and J. G. Desjardins, *J. Gas Chromatogr.*, 2 (1964) 270.
489 J. C. M. Schogt, P. H. Begemann and J. H. Recourt, *J. Lipid Res.*, 2 (1961) 142.
490 J. E. Schvarberg, R. E. Sievers and R. W. Moshier, *Anal. Chem.*, 42 (1970) 1828.
491 G. Schwedt and H. A. Ruessel, *Chromatographia*, 5 (1972) 242.
492 P. H. Scott, *J. Chromatogr.*, 70 (1972) 67.
493 W. G. Scribner, W. J. Treat, J. D. Weis and R. W. Moshier, *Anal. Chem.*, 37 (1965) 1136.
494 C. Segard, B. Roques, C. Pommier and G. Guiochon, *Anal. Chem.*, 43 (1971) 1146.
495 J. N. Seiber, D. G. Crosby, H. Fouda and J. Soderquist, *J. Chromatogr.*, 73 (1972) 89.
496 W. Seidenstücker, *Z. Anal. Chem.*, 237 (1968) 280.
497 N. P. Sen, *J. Chromatogr.*, 51 (1970) 301.
498 F. Sharokhi and C. W. Gehrke, *J. Chromatogr.*, 36 (1968) 31.
499 F. Sharokhi and C. W. Gehrke, *Anal. Biochem.*, 24 (1968) 281.
500 W. R. Sherman, S. L. Goodwin and M. Zinbo, *J. Chromatogr. Sci.*, 9 (1971) 363.
501 T. Shigematsu, M. Matsui and K. Utsunomiya, *Bull. Chem. Soc. Jap.*, 41 (1968) 763.
502 Y. Shimoishi and K. Tôei, *Talanta*, 17 (1970) 165.
503 A. T. Shulgin, *Anal. Chem.*, 36 (1964) 920.
504 J. P. Shyluk, C. G. Youngs and O. L. Gamborg, *J. Chromatogr.*, 26 (1967) 268.
505 R. F. Sieck, J. J. Richards, K. Iversen and C. V. Banks, *Anal. Chem.*, 43 (1971) 913.
506 E. R. Sievers, W. I. Connolly and W. D. Ross, in A. Zlatkis (Editor), *Advances in Chromatography 1967*, Chromatography Symposium, Houston, Texas, 1967, p. 104.
507 E. R. Sievers, B. W. Ponder, M. L. Morris and R. W. Moshier, *Inorg. Chem.*, 2 (1963) 693.
508 P. G. Simmonds, B. C. Pettitt and A. Zlatkis, *Anal. Chem.*, 39 (1967) 163.
509 P. G. Simmonds and A. Zlatkis, *Anal. Chem.*, 37 (1965) 302.
510 P. M. Simpson, *J. Chromatogr.*, 77 (1973) 161.
511 J. E. Sinsheimer and R. V. Smith, *J. Pharm. Sci.*, 56 (1967) 1280.
512 B. Sjöquist and E. Änggärd, *Anal. Chem.*, 44 (1972) 2297.
513 G. P. Slater, *J. Chromatogr.*, 64 (1972) 166.
514 J. Sliwick, T. Kowalska, J. Rzepa and A. Biernat, *Microchem. J.*, 18 (1973) 207.

515 J. H. Sloneker, in H. A. Szymanski (Editor), *Biomedical Applications of Gas Chromatography*, Vol. 2, Plenum Press, New York, 1968, p. 87.
516 B. Smith and O. Carisson, *Acta Chem. Scand.*, 17 (1963) 455.
517 E. D. Smith and H. Sheppard, *Nature (London)*, 208 (1965) 878.
518 E. D. Smith and K. L. Shewbart, *J. Chromatogr. Sci.*, 7 (1969) 704.
519 R. V. Smith and S. L. Tsai, *J. Chromatogr.*, 61 (1971) 29.
520 R. J. Soukup, R. J. Scarpellino and E. Danielczik, *Anal. Chem.*, 36 (1964) 2255.
521 C. S. Springer, D. W. Meek and R. E. Sievers, *Inorg. Chem.*, 6 (1967) 1105.
522 S. R. Srinivasan, B. Radhakrishnamurthy, E. R. Dalferes and G. S. Berenson, *Anal. Biochem.*, 35 (1970) 398.
523 D. L. Stalling, C. W. Gehrke and R. W. Zumwalt, *Biochim. Biophys. Res. Commun.*, 31 (1968) 616.
524 D. L. Stalling, G. Gille and C. W. Gehrke, *Anal. Biochem.*, 18 (1967) 118.
525 G. W. Stevenson, *Anal. Chem.*, 38 (1966) 1948.
526 W. Stoffel, F. Chu and E. H. Ahrens, *Anal. Chem.*, 31 (1959) 307.
527 J. Stouffer, P. I. Jaakonmäki and T. J. Wenger, *Biochim. Biophys. Acta*, 127 (1966) 261.
528 E. Stummpp, *Z. Anal. Chem.*, 242 (1968) 225.
529 W. R. Supina, in H. A. Szymanski (Editor), *Biomedical Applications of Gas Chromatography*, Vol. 1, Plenum Press, New York, 1964, p. 271.
530 W. R. Supina, R. F. Kruppa and R. S. Henly, *J. Amer. Oil Chem. Soc.*, 44 (1967) 74.
531 W. R. Supina, R. F. Kruppa and R. Henley, *J. Amer. Oil Chem. Soc.*, 44 (1967) 965.
532 C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
533 C. C. Sweeley and D. Vance, in G. Marrinetti, *Lipid Chromatographic Analysis*, Marcel Dekker, New York, 1967, p. 465.
534 W. H. Tallent and R. Kleiman, *J. Lipid Res.*, 9 (1968) 146.
535 S. Tamura, A. Suzuki, Y. Aoki and N. Otake, *Agr. Biol. Chem.*, 28 (1964) 650.
536 F. S. Tanaka and R. G. Wien, *J. Chromatogr.*, 87 (1973) 85.
537 M. Tanaka, T. Shono and K. Shinra, *Anal. Chim. Acta*, 43 (1968) 157.
538 J. O. G. Tatton and P. J. Wagstaffe, *J. Chromatogr.*, 44 (1969) 284.
539 T. A. Taulli, *J. Chromatogr. Sci.*, 7 (1969) 671.
540 M. L. Taylor and E. L. Arnold, *Anal. Chem.*, 43 (1971) 1328.
541 M. L. Taylor, E. L. Arnold and R. E. Sievers, *Anal. Lett.*, 1 (1968) 735.
542 J. B. Terrill and E. S. Jakobs, *J. Chromatogr. Sci.*, 8 (1970) 604.
543 K. Tesařík, S. Ghyczy and Y. S. Pansare, *Chromatographia*, 4 (1971) 396.
544 B. Teuwissen and A. Darbre, *J. Chromatogr.*, 49 (1970) 298.
545 B. Teuwissen, C. Lenain, C. Dorlet and J. Leonis, *J. Pharm. Belg.*, (1963) 81.
546 J. P. Thenot and E. C. Horning, *Anal. Lett.*, 5 (1972) 21.
547 J. P. Thenot and E. C. Horning, *Anal. Lett.*, 5 (1972) 905.
548 J. P. Thenot, E. C. Horning, M. Stafford and M. G. Horning, *Anal. Lett.*, 5 (1972) 217.
549 B. S. Thomas, *J. Chromatogr.*, 56 (1971) 37.
550 B. S. Thomas, C. Eaborn and D. M. R. Walton, *Chem. Commun.*, 13 (1966) 408.
551 B. S. Thomas and D. M. R. Walton, *J. Endocrinol.*, 41 (1968) 203.
552 K. Tsuji and J. H. Robertson, *Anal. Chem.*, 41 (1969) 1332.
553 K. Tsuji and J. H. Robertson, *Anal. Chem.*, 42 (1970) 1661.
554 E. O. Umeh, *J. Chromatogr.*, 51 (1970) 139.
555 E. O. Umeh, *J. Chromatogr.*, 51 (1970) 147.
556 E. O. Umeh, *J. Chromatogr.*, 56 (1971) 29.
557 W. J. A. VandenHeuvel, in M. B. Lipsett (Editor), *Gas Chromatography of Steroids in Biological Fluids*, Plenum Press, New York, 1965, p. 277.
558 W. J. A. VandenHeuvel, *J. Chromatogr.*, 27 (1967) 85.
559 W. J. A. VandenHeuvel, *J. Chromatogr.*, 36 (1968) 354.
560 W. J. A. VandenHeuvel and K. L. K. Braly, *J. Chromatogr.*, 31 (1967) 9.
561 W. J. A. VandenHeuvel, R. P. Buhs, J. R. Carlin, T. A. Jacob, F. R. Koniuszy, J. L. Smith, N. R. Trenner, R. W. Walker, D. E. Wolf and F. J. Wolf, *Anal. Chem.*, 44 (1972) 14.
562 W. J. A. VandenHeuvel, W. L. Gardiner and E. C. Horning, *J. Chromatogr.*, 18 (1965) 391.
563 W. J. A. VandenHeuvel and E. C. Horning, *Biochim. Biophys. Acta*, 74 (1963) 560.
564 W. J. A. VandenHeuvel and E. C. Horning, *Med. Res. Eng.*, 7 (1968) 10.

565 R. Varma, R. S. Varma and A. H. Wardi, *J. Chromatogr.*, 77 (1973) 222.
566 M. Vecchi and K. Kaiser, *J. Chromatogr.*, 26 (1967) 22.
567 R. Vilceanu and P. Schulz, *J. Chromatogr.*, 82 (1973) 279.
568 R. Vilceanu, P. Schulz, R. Draghici and P. Soimu, *J. Chromatogr.*, 82 (1973) 285.
569 M. Vilkas, H. Jan, G. Boussac and M. C. Bonnard, *Tetrahedron Lett.*, 14 (1966) 1441.
570 J. W. Vogh, *Anal. Chem.*, 43 (1971) 1618.
571 E. M. Volpert, N. Kundu and J. B. Dawidzik, *J. Chromatogr.*, 50 (1970) 507.
572 M. L. Vorbeck, L. R. Mattick, F. A. Lee and C. S. Pederson, *Anal. Chem.*, 33 (1961) 1512.
573 T. Walle, *Acta Pharm. Suecica*, 5 (1968) 367.
574 T. Walle and H. Ehrsson, *Acta Pharm. Suecica*, 7 (1970) 389.
575 T. Walle, G. Schill and J. Vessman, *Acta Pharm. Suecica*, 3 (1966) 167.
576 C. B. Warren and E. J. Malec, *J. Chromatogr.*, 64 (1972) 219.
577 E. Watson and S. M. Kalman, *J. Chromatogr.*, 56 (1971) 209.
578 J. R. Watson and P. Crescuolo, *J. Chromatogr.*, 52 (1970) 63.
579 R. B. Watts and R. G. O. Kekwick, *J. Chromatogr.*, 88 (1974) 15.
580 A. H. Weiss and H. Tambawala, *J. Chromatogr. Sci.*, 10 (1972) 120.
581 W. W. Wells, C. C. Sweeley and R. Bentley, in H. A. Szymanski (Editor), *Biomedical Applications of Gas Chromatography*, Vol. 1, Plenum Press, New York, 1964, p. 169.
582 R. E. Weston and B. B. Wheals, *Analyst (London)*, 95 (1970) 680.
583 F. Weygand, *Z. Anal. Chem.*, 205 (1964) 406.
584 F. Weygand, B. Kolb, A. Prox, M. A. Tilak and I. Tomida, *Hoppe-Seyler's Z. Physiol. Chem.*, 322 (1960) 38.
585 E. H. White and H. Scherrer, *Tetrahedron Lett.*, 21 (1961) 758.
586 J. C. N. Whyte, *J. Chromatogr.*, 87 (1973) 163.
587 M. Wilcox, *Anal. Biochem.*, 32 (1969) 191.
588 G. R. Wilkinson and E. L. Way, *Biochem. Pharmacol.*, 18 (1969) 1435.
589 C. M. Williams, *Anal. Biochem.*, 11 (1965) 224.
590 P. W. Wilson, D. E. M. Lawson and E. Kodicek, *J. Chromatogr.*, 39 (1969) 75.
591 M. K. Withers, *J. Gas Chromatogr.*, 6 (1968) 242.
592 W. R. Wolf, M. L. Taylor, B. H. Hughes, T. O. Tiernan and R. E. Sievers, *Anal. Chem.*, 44 (1972) 616.
593 R. Wood, *J. Gas Chromatogr.*, 6 (1968) 94.
594 R. D. Wood, P. K. Raju and R. Reiser, *J. Amer. Oil Chem. Soc.*, 42 (1965) 81.
595 H. H. Wotiz, G. Charransol and I. N. Smith, *Steroids*, 10 (1967) 127.
596 F. F. H. Wu, J. Götz, W. D. Samieson and C. R. Masson, *J. Chromatogr.*, 48 (1970) 515.
597 J. P. Zanetta, W. C. Breckenridge and G. Vincendon, *J. Chromatogr.*, 69 (1972) 291.
598 J. P. Zanetta and G. Vincendon, *J. Chromatogr.*, 76 (1973) 91.
599 D. Zarazir, P. Chovin and G. Guiochon, *Chromatographia*, 3 (1970) 180.
600 M. Zinbo and W. R. Sherman, *Tetrahedron Lett.*, 33 (1969) 2811.
601 D. F. Zinkel, M. B. Lanthrop and C. Zank, *J. Gas Chromatogr.*, 6 (1968) 158.
602 C. Zomzely, G. Marco and E. Emery, *Anal. Chem.*, 34 (1962) 1414.
603 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 55 (1971) 267.
604 T. S. Zvarova and I. Zvara, *J. Chromatogr.*, 49 (1970) 290.

Author Index (Chromatographic Review articles)

Subject Index (Chromatographic Review articles)

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journal of
chromatography news section

APPARATUS

N-757

SWITCHING AND SAMPLING VALVES FOR LC

A series of rotary Teflon® switching and sampling valves is available from Rheodyne Offered in both manual and automatic models, the new valves are chemically inert, carry zero dead volume, work up to 300 p.s.i., and are available in either 0.8- or 1.5-mm bore.

Applications for the Type 50 Teflon® rotary valves include chromatography, sample injection, column switching, recycling, reagent switching, fraction collection, stream sampling and quantitative reagent injection. Double 3-way valves, 4-way valves to interchange two streams, 6-position valves to select any one of six streams, and an injection valve supplied with a 5-ml sample loop and luer connector are available together with larger bore valves (1.5 mm).

N-762

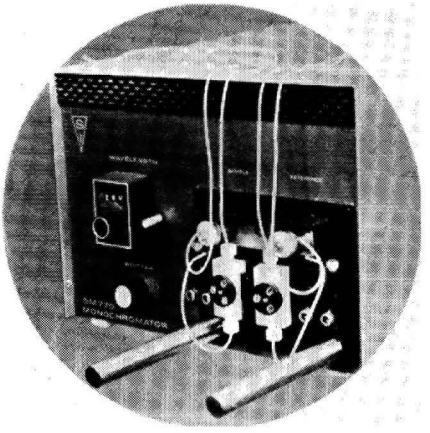
TRACOR GAS CHROMATOGRAPH

Reprints of the article "Design concepts of a digital gas chromatograph" are available from Tracor, describing the development, operation procedure and performance of the Tracor Model 560 gas chromatograph.

N-760

QUICK-CHANGE FITTINGS FOR PHOTOMETRIC LC DETECTOR

The Model SF 770 continuously variable wavelength monitor for LC, from Schoeffel, is equipped now with quick-change Teflon®-type fittings for cell and column interface. The cell body is made of like material. The researcher can use the monitor with several different column systems without time-consuming interface

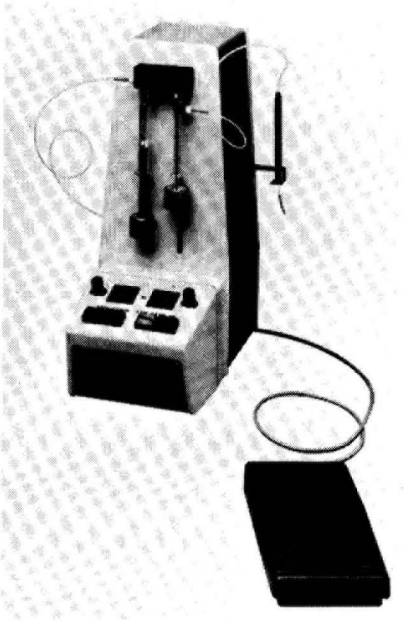


preparations. Because of the quick-change ability, the SF 770 can also be placed in series with fixed-wavelength detectors to verify results. The tubing is quite flexible so that troublesome cutting and bending is avoided. All the connections are of low dead volume to insure bubble-free operation.

N-761

DIGITAL DILUTER/DISPENSER

A digital diluter and dispenser for micro dilutions with a high degree of accuracy and speed is available from Hamilton. The system uses digital input settings, digital electronics and stepping motors. Because it requires so little sample to make precise dilutions, the digital diluter can be used to perform micro chemistries with valuable liquids such as radioactive cocktails, or with samples of limited availability. The dead volume of the diluter is small when changing from one solution to another.



An advantage of the system is that all the syringes can be interchanged in less than 20 sec, and that it is fully inert, and may handle samples as low as 1 μl with dilutions as high as 10 ml. A valve block option converts the instrument into a dual dispenser in about 30 sec having dispensing volumes between 1 μl and 10 ml. Easy-to-read digital volume settings are made step-wise from 1–99% of syringe volume for each syringe position. An electronic motor drives the syringe plunger to dispense the exact volume set. The system operates without liquid leaks at temperatures as low as 0°.

Other characteristics include automatic zero setting, smooth starting and stopping of the motors, hand- or foot-switch operation, and overload protection. Immunity to electrical noise is built into the system by means of C-MOS logic. The device also has the ability to operate in conjunction with other equipment.

All Hamilton TLL-type syringes from 1705-TLL to 1010-TLL can be used with both the diluter and dispenser. D-Type sample syringes with a side-arm for gas-free operation are available for the diluter.

N-776

HIGH-SENSITIVITY UV–VIS SPECTROPHOTOMETER

A bulletin describing the Schoeffel Model HS 870 high-sensitivity UV–visible spectrophotometer has appeared. Instrument features are carefully detailed and reference is made to the many and varied applications for which this instrument can be used. Particular attention is given to the conversion features of this instrument whereby it can be easily used for HPLC applications as well.

N-764

FLUORESCENCE DETECTOR

A technical bulletin describing the Model FS 970 fluorescence detector for LC is available from Schoeffel. The bulletin provides data on the instrument's performance and stresses the advantage of using a continuously variable wavelength source.

N-763

UV MONITOR FOR LC

Analabs announces the UV monitor 254-280. It is a sensitive, dual-beam, flow-through UV detector designed for use in HPLC. Read-out of absorbance from the Analabs UV monitor is transmitted to the recorder on a linear scale. This permits direct quantitation from the curves drawn on the recorder charts.

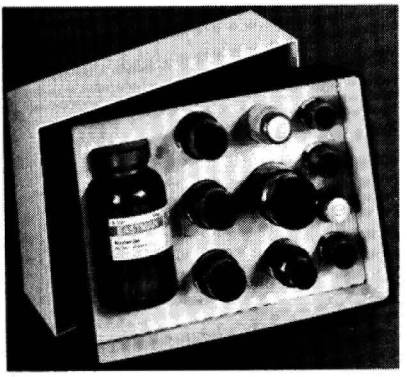
CHEMICALS

N-769

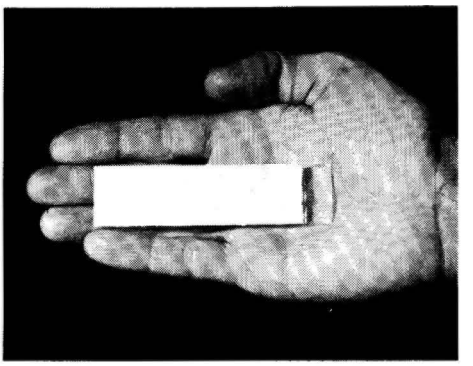
ELECTROPHORESIS REAGENT SETS

Four sets of reagents for polyacrylamide gel electrophoresis are available from Eastman Kodak. All four sets contain reagents grouped together for specific applications. In addition, each set includes an illustrated instruction manual with an introduction to electrophoresis, a guide to sample preparation and a bibliography.

The standard acrylamide electrophoresis reagent set (13201) contains acrylamide, N,N′-methylenebisacrylamide, ammonium persulfate, TEMED, riboflavin, tracker dye and stain. The solubilizable gel electrophoresis reagent set (13203) contains N,N′-diallyltartardiamide as the cross-linking agent for the preparation of solubilizable acrylamide gels. Periodic acid dissolves the gels with relative ease and no viscous residue, in preparation for liquid scintillation counting. SDS electrophoresis reagent set (13204) includes SDS together with the acrylamide, N,N′-methylenebisacrylamide, TEMED, ammonium persulfate, mercaptoethanol, and stain for protein molecular-weight determinations.



Finally, the Eastman 13202 electrophoresis starter reagent set (see photograph), combines all of the above reagents in the other three sets. The four sets provide the necessary reagents in approximately the correct proportions for a variety of separations.



N-768

MICROSCOPE-SLIDE-SIZE TLC PLATE

Analabs have available a 2.5 x 10 cm microscope-slide-size TLC plate. This miniature plate is supplied as a 10 x 20 cm glass plate, prescored on the uncoated side to make eight "microscope-size" plates. These TLC plates are designed for the quick, economical monitoring of reactions. They require the minimum amount of solvent.

N-758

PRE-COATED PLATES AND SUPPLIES

Kontes announces the availability of TLC precoated plates and supplies, described in a catalog which has sections covering the elements of TLC technique, reagent specifications data, and a toxicology system. It also lists an assortment of TLC plates, reagents and supplies available from the company.

N-772

CHROMATOGRAPHY CATALOGUE

"Chromatography Electrophoresis and Membrane Technology" (Catalogue Z, 1974–75, 94 pages) from Bio-Rad Labs., has sections on chromatography (ion-exchange, gel, affinity, adsorption, and columns and fittings); electrophoresis (reagents, equipment and systems); and membrane technology (hollow fibre devices and accessories).

N-766

GLASS CAPILLARY COLUMNS

Individually tested coated glass capillary columns have been introduced by LKB. Columns are available with several stationary phases, being insensitive to overloading and having high temperature stability.

PROCEDURES

N-773

CAMAG BIBLIOGRAPHY FOR TLC

Camag's "TLC Bibliography Service" (CBS 36) contains 243 references on papers dealing with TLC, divided into classes of compounds or fields of application. In the product section information is given on the ELUCHROM, the Chromatocharger, the T-scanner and the VARICOL fraction collector.

N-774

GAS-CHROM NEWSLETTER

Gas-Chrom Newsletter (Vol. 16, No. 4) from Applied Science Labs. describes various new products and applications: Methylamine analysis with Pennwalt 231 GC packing; audio-visual courses in analytical chemistry (basic gas chromatography, infrared spectroscopy, atomic adsorption spectroscopy, and NMR spectroscopy); precision-bore HPLC tubing; estrone metabolites; gas analysis by GC with carbon molecular sieve packing; acyl transferase and lecithin:cholesterol acyl transferase (LCAT) substrates.

N-771

CHROMATOGRAPHY BULLETIN

"Chromatography Notes" from Waters Ass. has sections on the variable-wavelength Model 440 UV detector, brewing applications of LC, identification of amino acids, details of the company's column packing materials catalogue, quality control of plastics, details of a LC teaching programme and finally the determination of slip agents in polyethylene films by LC.

NEW BOOKS

Grundlagen und Methoden der chemischen Emissionsspektralanalyse, by R. Mannkopff and G. Friede, Verlag Chemie, Weinheim, 1975, x + 218 pp., 128 figs., 17 tables, price DM 78.00.

Organische Analyse unter besonderer Berücksichtigu von Arzneistoffen, (thorough revision of Bauer/Moll, Die organische Analyse), by R. Pohloudek-Fabini and T. Beyrich, Akademische Verlagsgesellschaft, Leipzig, 1975, *ca.* 736 pp., price DM 49.00.

Ozone chemistry and technology; A review of the literature 1961–1974, edited by J.S. Murphy and J.R Orr, Franklin Institute Press, Philadelphia, Pa., 1975, vii + 392 pp., price US$ 30.00.

Chromatographic analysis of the environment, edited by R.L. Grob, Marcel Dekker, New York, 1975, xi + 734 pp., price US$ 49.50, ISBN 0-8247-1259-5.

The interpretation of infrared spectra, A programmed introduction, by R.R. Hill and D.A.E. Rendell, Heyden & Son, London, 1975, xii + 196 pp., price £ 4.40, US$ 12.00, DM 36.00, ISBN 0-85501-066-5.

More spectroscopic problems in organic chemistry, by A.J. Baker, with the collaboration of T. Cairns, G. Eglinton and F.J. Preston, Heyden & Son, London, 2nd ed., 1975, vii + 127 pp., price £ 2.50, US$ 6.90, DM 20.50, ISBN 0-85501-097-5.

Isoelectric focusing, edited by J.P. Arbuthnott and J.A. Beeley, Butterworths, London, 1975, xi + 367 pp., price £14.45, ISBN 0 408 70659 7.

Analysis of silicones, edited by A.L. Smith, Wiley, Chichester, 1975, 416 pp., price £14.60.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

PUBLICATION SCHEDULE FOR 1975

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	D 1974	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	101/1 101/2 102	103/1 103/2 104/1	104/2 105/1 105/2	106/1 106/2	107/1 107/2	108/1 108/2	109/1 109/2	110/1 110/2	111/1	111/2	112	114/1 114/2	
REVIEWS*				113/1					113/2		113/3		

* Volume 113 will consist of *Chromatographic Reviews*. The issues comprising this volume will not be published consecutively, but will appear at various times in the course of the year.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. (a) Original research work not previously published in a generally accessible language in other periodicals (Full-length papers). (b) Review articles. (c) Short communications and Notes. (d) Book reviews; News; Announcements. (e) Bibliography of Paper Chromatography, Thin-Layer Chromatography, Column Chromatography, Gas Chromatography and Electrophoretic Techniques. (f) Chromatographic Data.

Submission of Papers. Three copies of manuscripts in English, French or German should be sent to: Editorial office of the Journal of Chromatography, P.O. Box 681, Amsterdam, The Netherlands. For *Review articles*, an outline of the proposed article should first be forwarded to the Editorial office for preliminary discussion prior to preparation.

Manuscripts. The manuscript should be typed with double spacing on pages of uniform size and should be accompanied by a separate title page. The name and the complete address of the author to whom proofs are to be sent should be given on this page. Authors of papers in French or German are requested to supply an English translation of the title. A short running title of not more than 50 letters (including spaces between the words) is also required for Full-length papers and Review articles. All illustrations, photographs, tables, etc., should be on separate sheets.

Heading. The title of the paper should be concise and informative. The title should be followed by the authors' full names, academic or professional affiliations, and addresses.

Summary. Full-length papers and Review articles should have a summary of 50–100 words. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes will be published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Particular attention should be paid to the size of the lettering to ensure that it does not become unreadable after reduction. Sharp, glossy photographs are required to obtain good halftones. Each illustration should have a legend, all the *legends* being typed together on a *separate sheet*. Coloured illustrations are reproduced at the author's expense.

References. References should be numbered in the order in which they are cited in the text and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places using superscript numerals. In the reference list, periodicals[1], books[2], and multi-author books[3] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Vol. 5, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*.

Proof. Two sets of proofs will be sent to the author to be carefully checked for printer's error s. Corsrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography, Elsevier Scientific Pu blishing Company, P.O. Box 330, Amsterdam, The Netherlands.

Subsc ription orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, Amsterdam, The Netherlands.

Publication. The *Journal of Chromatography* (including *Chromatographic Reviews*) appears fortnightly and has 14 volumes in 1975. The subscription price for 1975 [Vols. 101–114 and Supplementary Vol. 4 (Bibliography of Electrophoresis 1968–1972)] is Dfl. 1365.00 plus Dfl. 120.00 (postage) (total US$ 631.91). Subscribers in the U.S.A., Canada and Japan receive their copies by air mail. Additional charges for air mail to other countries are available on request. Back volumes of the *Journal of Chromatography* (Vols. 1 through 100) are available at Dfl. 100.00 (plus postage).

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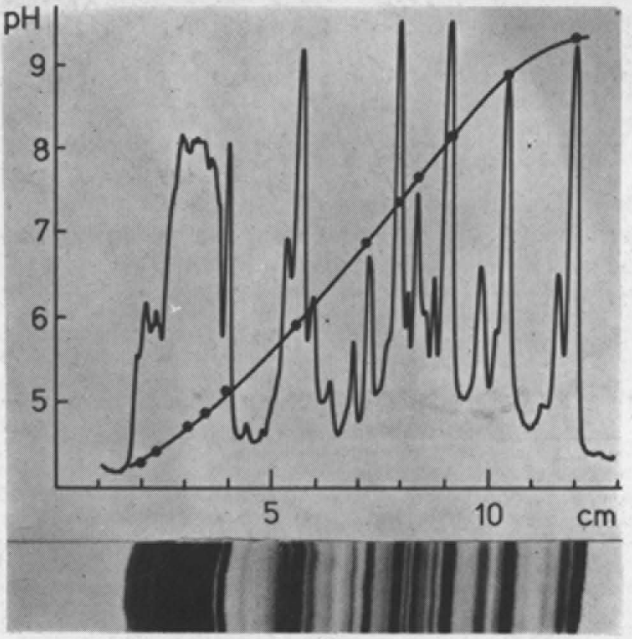

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