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edited by

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CONTENTS

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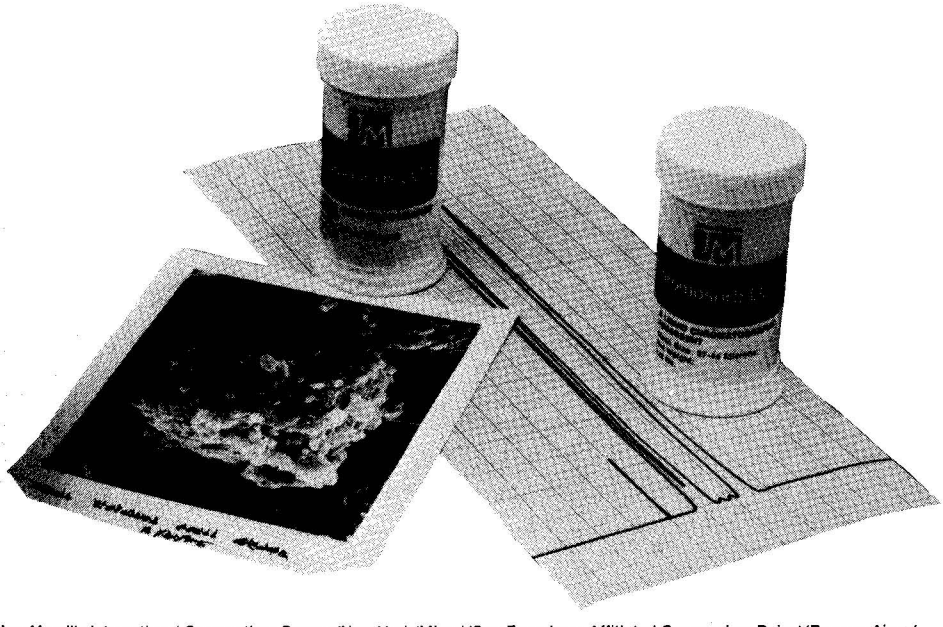

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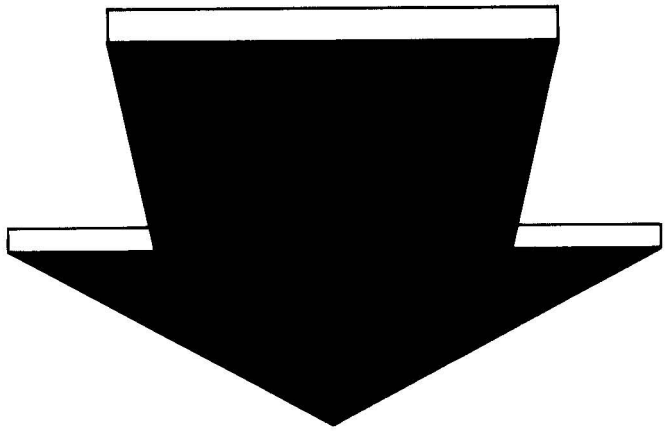

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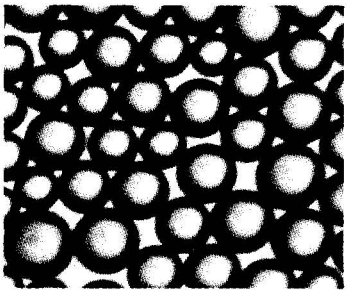

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Sample	Type H (0.1 μmole) + Norleucine (0.05 μmole)
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Ninhydrin Flow Rate	35 ml/hr
First Buffer	3.49 ± 0.01 (0.20 N)
Second Buffer	4.30 ± 0.02 (0.20 N)

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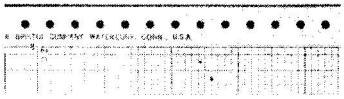

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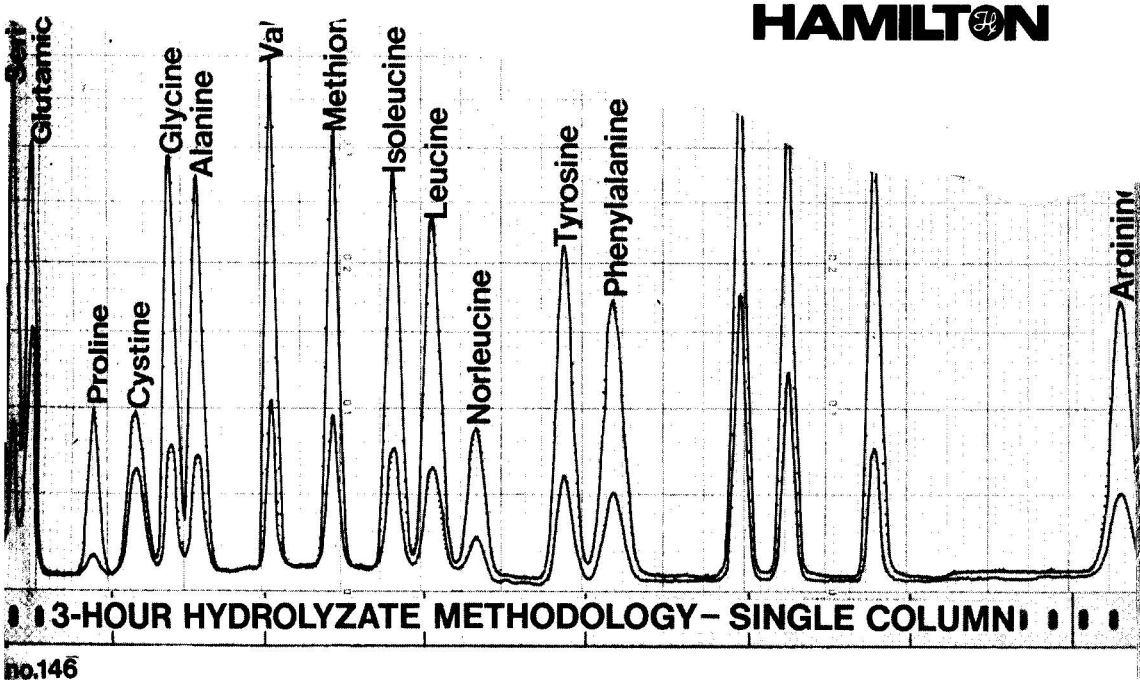

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Chromatographic Reviews

CHREV. 94

ADVANCES IN SEPARATION TECHNIQUES IN SEQUENCE ANALYSIS OF PROTEINS AND PEPTIDES

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(Received February 16th, 1976)

CONTENTS

1. INTRODUCTION

In spite of numerous efforts to overcome the time-consuming operations in sequence analysis, either by automation of current procedures or by outlining new methods suitable for computerization, chromatographic methods continue to be the keystone in the multi-step procedures used for the determination of the primary structure of proteins and peptides.

During the past 30 years, numerous methods have appeared that are suitable for the determination of the liberated N-terminal amino acid after applying Edman's classical single-step procedure. About four years ago, together with the late Jan Rosmus, we tried to summarize the data available on chromatographic separations of different

derivatives used in sequence analysis and found that there were about 41 types of derivative (Rosmus and Deyl[1,2]). At first sight it was obvious that the individual methods were developed to considerably different degrees and also that the separation techniques used for individual types of derivative differed substantially in respect to the quality of separations obtained. Some of these techniques were very popular and necessitated the division of the review into two parts, one of which was devoted solely to the separation of dinitrophenyl (DNP) derivatives and phenylthiohydantoin (PTH) derivatives, while the other was devoted to the remaining 39 methods.

Now, four years later, it is, perhaps, reasonable to re-examine this topic. The above division of the problem into two parts according to the popularity of individual techniques seems justified even today; in the meantime, however, the interests of most research workers have changed. The separation of dinitrophenyl derivatives appears nowadays to be only of historical interest and these were replaced by fluorescent derivatives, mainly the 1-dimethylaminonaphthalene-5-sulphonyl (dansyl; Dns) compounds, which offer much higher sensitivity and are capable of giving very good separations. Unlike DNP-amino acid derivatives, hydantoins, phenylthiohydantoins and other derivatives of this type withstood the fashionable trends over the years and interest in their use in facilitating separations remains high. This latter fact is obviously due to the use of Edman's procedure, which, except for being automated, has not changed substantially. Currently, progress in chromatographic techniques in the area of sequence analysis appears to be intensive rather than extensive. Since the first steps taken in the chromatography of Dns-amino acids about 10 years ago (Seiler and Wiechman[3] and Deyl and Rosmus[4]), the technique for their separation has been highly developed and today offers a wide selection of all types of separation, including the flat-bed arrangement, classical liquid-column separation and high-speed, high-resolution chromatography; the same considerations hold for different organic isocyanates, especially PTH-amino acids.

As mentioned earlier, the newly developed methods are few, and most are directed towards slight modifications of aromatic sulphonyl chlorides, which would offer higher yields of fluorescence and thus increase the sensitivity of the method beyond the level of Dns derivatives. Among the really new procedures, the use of pivalyl and benzoyl chloride for the gas chromatographic separation and determination of N-terminal amino acids should be emphasized (Cavadore *et al.*[5]).

It is not within the scope of this paper to present an exhaustive review on the chromatographic separations of N-terminal amino acid derivatives and those readers who require more detailed information should be directed to previously published reviews, many of which have lost little of their value even after the many years that have elapsed since they appeared (Fox[6], Rosmus and Deyl[1,2], Meloun[7], Holeyšovský[8], Pataki[9], Bailey[10], Seiler[11], Gray[12], Deyl and Juřicová[13], Deyl[14]). It is therefore desirable to concentrate on the progress that has been made within the last four years respecting the effects of the new progressive chromatographic techniques, which involve not only the quality of separation but equally the problems of quantitation.

In the formal presentation of this survey, repetition of the mechanisms of the reactions examined in the two previous reviews (Rosmus and Deyl[1,2]) is avoided. Thus only the reaction schemes of the newly introduced types of reagents are presented here. Reaction schemes of analogous reagents, which have generally the same structural skeleton but differring only in one or two substituents have also been omitted.

2. DNS DERIVATIVES

(A) Derivatization

In principle, the technique of dansylation has not changed and most workers recommend that the reaction be carried out in 0.1 *M* $NaHCO_3$. In addition to the techniques described in our previous reports, it would perhaps be reasonable to refer here to the method of Varga and Richards[15], which, when carefully carried out, allows the quantitation of Dns-amino acids after polyamide thin-layer chromatography (TLC) at the picomole level.

To 3.6×10^{-11} mole of the protein to be analysed, contained in a test tube, 5 μl of 0.1 *M* $NaHCO_3$ are added and made to cover the base of the tube by vibrating it on a Vortex mixer; 5 μl (approx. 500 molar excess) of Dns chloride (Dns-Cl) solution (2 mg/ml) in acetone are then added and, after briefly mixing it, the reaction mixture is allowed to react for 12 h at 20°. The mixture is hydrolysed with 6 *N* HCl, the residue obtained after evaporation dried *in vacuo* and the Dns-amino acids and side products of the reaction are then extracted by vibrating the test tube with 25 μl of ethyl acetate saturated with water. The extract is spotted on two polyamide layers (2 μl each) such that the spots on the starting line are less than 2 mm in diameter. Simultaneously, a standard mixture of Dns-amino acids is run in parallel, thus providing an internal control for accurate quantitation, as it eliminates differences in TLC plate batch behaviour, humidity, solvents and minor discrepancies in the chromatographic process itself.

For hydrolysis under the above conditions, 25 μl of 6 *N* HCl are used and the evaporated residue after hydrolysis taken up in pyridine–acetic acid (1:1). Different amino acid derivatives exhibit different levels of stability towards hydrolysis, as summarized by Casola *et al.*[16] (Table 1).

TABLE 1

RECOVERY OF Dns-AMINO ACIDS AFTER TREATMENT WITH 6 *N* HYDROCHLORIC ACID AT 110° FOR 16 h

Amino acid	*Recovery (%)*
Lys	92
Phe	93
Leu	92
Ile	86
Val	93
Pro	14
Ser	65
Thr	97
Asp	93
Glu	100
Gly	64
Ala	87

(B) Flat-bed separations

As mentioned in many earlier papers, one of the considerable problems in Dns-amino acid chromatography is that the unreacted Dns-Cl hydrolyses to the corre-

sponding acid, which interferes with the chromatographic separation if the choice of the solvent is such that the free acid has a high R_F value. Varga and Richards[15] recommend overcoming this problem by multiple development with the solvents systems used by Woods and Wang[17], *i.e.*, benzene–acetic acid (9:1), formic acid–water (1.5:98.5 or 15:100) and later ethyl acetate–methanol–acetic acid (20:1:1). With the first system the Dns acid remains on the starting line and does not interfere in the subsequent step.

Most of the Dns-amino acids are resolved by running the sample in parallel in the first two solvent systems. Dns-arginine (Arg) is separated from Dns-histidine (His), and Dns-aspartic acid (Asp) from Dns-glutamic acid (Glu), by running the plate developed previously in benzene–acetic acid (9:1) in the last solvent system, *i.e.*

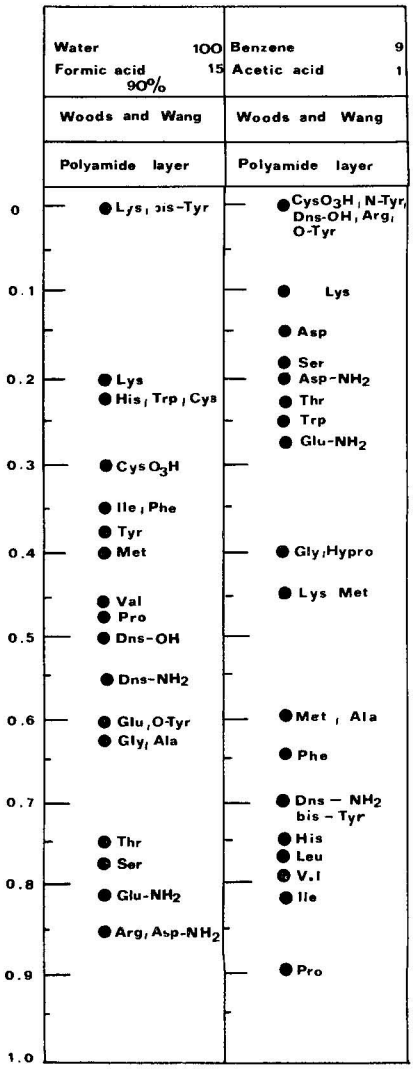


Fig. 1. Schematic representation of spots of Dns derivatives of amino acids on polyamide layer (Woods and Wang[17]). These data are used for the first run separation using the technique of Varga and Richards[15] (for details see text).

ethyl acetate–methanol–acetic acid (20:1:1). In order to separate Dns-Arg, Dns-serine (Ser) and Dns-threonine (Thr), the ethyl acetate–methanol–acetic acid mobile phase is run over the plate developed previously in formic acid–water (1.5:98.5 or 15:100). Positions of individual spots are summarized in Fig. 1. Perhaps the most unique feature of this procedure lies in the use of polyamide layers that are covered on both sides with the sorbent. Usually, the reference mixture is spotted on the reverse side of the layer. This arrangement is necessary for subsequent quantitation, as will be described later.

The only disadvantage of the above procedure is that it constantly obscures the spot of Dns-cysteine (Cys), which is indistinguishable from that of the free acid. It is therefore recommended that some of the solvent systems that offer almost complete separation of all Dns-amino acids, as evolved by Deyl and Rosmus[4] and Arnott and Ward[18], be applied.

Quantitation of the spots can be achieved by direct scanning of emitted fluorescence as reported by Seiler[11] for silica gel plates. The fluorescence depends, however, on the wetness of the plate, and apparently some decomposition or evaporation of the Dns derivatives occurs even if the plates are stored in a completely dark place. In the recently reported method of Varga and Richards[15], suitable for polyamide layers, the heating procedure of Boulton *et al.*[19], which increases the sensitivity of detection, is omitted. Essentially, the procedure of Varga and Richards[15] is a more precise application of the original trials of Bruton and Hartley[20], Weiner *et al.*[21] and Spivak *et al.*[22] for quantitative microanalysis.

In addition to direct *in situ* spectrofluorimetry, Varga and Richards[15] described the possibility of quantitation of fluorescent spots by photocopying densitometry. This method is somewhat more laborious than direct scanning; however, it offers the advantage that permanent records can be kept. In principle, a sandwich is made by using contrast film on one side of a gelatin filter (Kodak Wratten No. 93) and a double-sided polyamide thin-layer plate on the other, with the layer containing the Dns-amino acid spots on the outside, thus directly facing the ultraviolet (UV) source. The plate transmits the visible light but prevents penetration by UV radiation. At the concentrations used for quantitation, the Dns-amino acids on the inside layer of the double-sided polyamide plate do not receive enough UV light to interfere with quantitation. The interference limit of these spots is a 1000-fold excess compared with the quantitated amount on the outside layer. The sandwich, held firmly together with appropriate clamps, is exposed to the UV lamp (UVS-11 minerallight) for 10 sec from a distance 17.5 cm. The sandwich is then taken apart and the film developed and scanned in an appropriate densitometer. Normally, quantitation is carried out at the 5–20 pmole range; however, the sensitivity extends down to the fentomole level. A further increase in sensitivity can perhaps be achieved by varying the excitation wavelength of fluorescence. The corresponding fluorescence intensities are now available, as indicated in Table 2.

In sequence analysis, not only quantitation but also identification may cause considerable problems. To facilitate identification the recent suggestion by Lederer that layers with different sorbent paths should be used with a single-solvent system seemed promising and resulted in the data reported by Deyl and Rosmus[23]. The spreading device has been divided into three equal parts and silica gel (silica gel. Woelm 210, neutral, 67 g per 100 ml of water), aluminium oxide (aluminium oxide

TABLE 2

FLUORESCENCE EMISSION MAXIMA AND RELATIVE FLUORESCENCE INTENSITY OF Dns-AMINO ACIDS ON POLYAMIDE PLATES

Amino acid	*Maximum (nm)*	*Intensity*	
		(μA)	*(%)*
Ala	470	0.50	100
Asn	470	0.59	118
Asp	468	0.44	88
Arg	468	0.48	96
Gln	466	0.45	90
Glu	469	0.54	108
Gly	470	0.43	86
Ile	465	0.47	94
Leu	468	0.54	108
Lys (ε-Dns)	470	0.42	84
Lys (ε-Dns)	469	0.53	106
Lys (bis-Dns)	468	0.70	140
Met	468	0.46	92
Phe	468	0.51	102
Pro	465	0.40	80
Ser	468	0.45	90
Thr	468	0.43	86
Tyr (α-Dns)	468	0.46	92
Tyr (bis-Dns)	405	0.33	118
	485	0.26	
Trp	466	0.47	94
Val	466	0.52	104
Dns–NH_2	395	0.40	80
Dns–OH	426	0.89	178

G, Woelm 113, 67 g per 100 g of water) and polyamide (polyamide, Woelm 410, 11 g per 100 ml of methanol) were used and spread in the individual parts of the device. The layers of silica gel and alumina were spread together in the first run of the spreader. The plates were then heated to 105° for 3 h and the second movement of the spreader was used to prepare the layer of polyamide. After being spread with polyamide, the layer was allowed to stand at room temperature for at least 15 min and finally dried again at 60° in a ventilated oven. The layers were approximately 250 μm thick and were stored in a desiccator over silica gel. Cellulose was not used for layer preparation as Dns derivatives tend to stick to the starting line and tail badly in all of the solvent systems used.

Among the solvent systems tested, the following two proved suitable for the identification procedure: chloroform–benzyl alcohol–acetic acid (70:3:3) (Fig. 2a); and *n*-butanol–pyridine–acetic acid–water (30:20:6:24) (Fig. 2b). As shown in the figures both systems allow complete identification of eighteen common acids in the form of their Dns derivatives. The usefulness of this technique becomes clear from some examples: in the solvent system based on chloroform the application of the third sorbent (polyamide) permits the identification of Asp, Arg and His in the presence of each other, a combination that is otherwise very difficult to separate. Valine (Val), and isoleucine (Ile) is another combination in which, without using the third sorbent

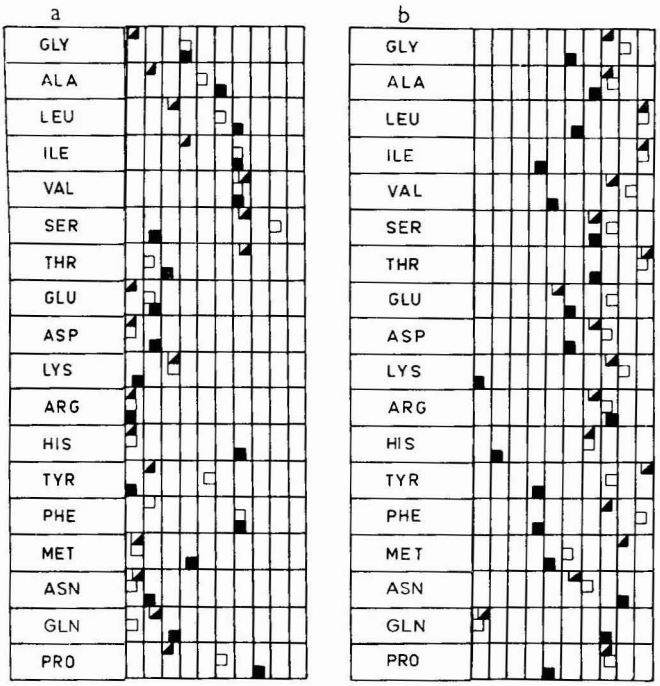


Fig. 2. Identification scheme for eighteen common amino acids as their Dns derivatives. Solvents: (a) chloroform–benzyl alcohol–acetic acid (70:30:3) and (b) *n*-butanol–pyridine–acetic acid–water (30:20:6:24). Sorbents used: aluminium oxide (hatched squares), silica gel (open squares), and polyamide (filled squares).

(aluminium oxide), the components cannot be distinguished. In the aqueous system (see Fig. 2b), similar combinations are Asp–lysine (Lys), Lys–Arg–proline (Pro) or glycine (Gly)–alanine (Ala). In order to facilitate identification, a simple system for describing the three spots relating to a particular amino acid derivative has been used: assuming that the accuracy of an R_F determination lies in the range of 0.05 R_F, each spot on a particular sorbent is characterized by the nearest R_F value ending in 0 or 5 (0.05, 0.10, 0.15, etc.). These help to make the system handy without using many layers for record purposes.

An important way to increase the sensitivity of an N-terminal amino acid determination is to make use of ^{14}C-Dns-Cl. This technique, developed by Casola *et al.*[16], offers a 50- to 100-fold increase in sensitivity over the conventional procedure. In combination with the precise separation procedure itself, it is possible to approach the picomole level. Quantitation is carried out with cut-out spots and the separation itself is two-dimensional with two subsequent runs in the second direction carried out with different solvent systems. A polyamide layer serves as sorbent. Aqueous formic acid (1.5%) has been used in the first dimension while benzene–acetic acid (90:10) served as the mobile phase for the first run in the second direction. Ethyl acetate–methanol–acetic acid (20:1:1) was used for re-running the chromatogram in the second dimension. This last run is sometimes referred to as a third dimension run.

Last, but not least, is the flat-bed procedure, which has been developed recently in the field of Dns derivatives in the so-called TLC on pre-loaded silica gel sheets. This technique is based on the original finding of Geiss *et al.*[24] that interactions between the mobile phase and ambient atmosphere may considerably influence final separation. Reasonably good separations were achieved with a formic acid gradient used for pre-loading (5–40%), provided that 10% of methanol was added to the pre-loading mixture. In some other cases the pre-loading gradient was extended so that it covered 0–40% of formic acid, but the presence of methanol was always necessary in order to depress tailing. Jänchen[25], who introduced this technique, stressed its theoretical value more than the practical application in sequence analysis, so that at present this procedure indicates only a perspective for structural studies and can hardly be exploited for routine work.

Although used only for the determination of the N-terminal amino acid in a single step, the method of Kato *et al.*[26] should be mentioned here, as it offers the possibility of estimating N-terminal amino acids in a mixture of proteins and peptides and, moreover, makes it possible to check small amounts of a contaminant in an oligopeptide.

In principle, a mixture of peptides labelled with Dns-Cl is separated by electrophoresis by using a 12.5% gel in 0.1 *M* Tris–acetate buffer (pH 8.2) that is 8 *M* in urea. After electrophoresis, gels are removed from the tubes by the usual procedure and fluorescent zones are cut off, homogenized and extracted with 5 ml of water at 37° for 16 h under stirring. In some instances the removal of the Dns peptides is incomplete under these conditions, in which event the following procedure is recommended: the gel section, containing labelled peptide is placed on the bottom of a new casting tube and the electrode buffer is layered on the gel. An electric current is then applied in the direction opposite to that used during separation. Usually electrophoresis for 90 min at 5 mA per gel is sufficient to transfer the sample completely to the inner buffer. The extract obtained by either of the two methods is lyophilized and washed three times with 1 ml of acetone in order to remove urea and sodium dodecyl sulphate (SDS). Under these conditions the Dns peptide remains precipitated without much loss. The precipitate is spun off, dried in a stream of nitrogen, dissolved in 0.5–1.0 ml of 5.7 *N* HCl and transferred to a hydrolysis tube. Hydrolysis is carried out at 110° for 4–6 h, the hydrolyzate is dried *in vacuo* within 30 min and the residue taken into solution with 0.5 ml of acetone. The extract is concentrated in a stream of nitrogen, if necessary, and subjected to TLC in the system adopted by Woods and Wang[17] using polyamide sheets. Dns–Tris occurs as a specific by-product during this procedure, and is characterized by having an R_F value of 0.95 in 1.5% aqueous formic acid on polyamide sheet.

(C) Liquid column chromatography and high-speed separations

In the renaissance of liquid column chromatography it would be rather surprising if a series of compounds such as the Dns derivatives of amino acids were not subjected to this type of separation. A procedure, using a polyamide column, was evolved by Deyl and Rosmus[27] and can briefly be characterized as follows. The column used had dimensions of 100 × 1 cm and was adjusted for constant-temperature operation (35°); it was filled with Woelm polyamide (15 g). As the degree of separation

obtained is considerably influenced by the method of column packing used, a special device developed by Kesner[28] for uniform column filling was adopted.

After the column had been filled, benzene was pumped through it for about 1 h in order to pack it. During the packing procedure, the flow-rate was maintained at 2.5 ml/min and while operating the column the flow-rate was decreased to 0.1 ml/min. Also during the packing procedure, the thermostat was set at 35° and the column, the mixing chamber and the reservoirs were adjusted to this temperature. The outlet of the column was connected to an adapted Farrand spectrofluorimeter cell; as in most instances the fluorescence intensity was much too high for the recorder scale, a proportionating pump was inserted. The excess of outflow from the column was either discarded or retained in a fraction collector for further investigation by flat-bed techniques. The fluorescence wavelengths were set to 340 and 500 nm for excitation and luminescence, respectively. The Farrand spectrofluorimeter was alternatively set for a decreased sensitivity (1.0 position on the sensitivity scale), and the proportionating pump was by-passed. The outlet flow was diluted with acetone or methylcellosolve from an additional reservoir. The individual parts used for the split-stream procedure and all the tubing used were parts of the Technicon amino acid analyzer. The overall asembly of the apparatus is presented in Fig. 3.

The measuring cuvette was adapted from a 5-mm round-shaped quartz tube, and the spectrofluorimeter gear box adapted to give a lower speed (20 cm/h).

The amount of sample analyzed varied from 50 to 500 μl. As the solvent system used by Woods and Wang[17] in thin-layer chromatography did not result in complete resolution of all the amino acid peaks, different proportions of benzene and acetic acid were examined. The most generally applicable mixture was that of benzene–acetic acid (90:5), in which, however, the fast-moving peaks of leucine (Leu) and isoleucine were not separated. In order to improve this situation, elution was started with a benzene/benzene–acetic acid (9:1) gradient, composed of two 200 ml mixed reservoirs. After 300 min, the inlet was switched automatically to the 9:1 benzene–acetic acid mixture and elution was carried out for the next 800 min without a gradient. In the final stage, this eluent was suddenly changed to a benzene–acetic acid (6:4) mixture, which made it possible to elute asparagine (Asn), hydroxyproline, arginine, cysteine and cysteic acid. The bluish band of Dns-amide remained uneluted and was removed during the regeneration procedure.

Before use, the column was washed with dry acetone (drying for 1.5 h was satisfactory), the flow-rate of the washing fluid being 1.5 ml/min. Acetone was then replaced with benzene, which was passed through the column for an additional 2 h. After this period, the column was ready for use for a fresh separation.

The gradient-elution system exhibits several advantages compared with the widely used flat-bed techniques. Firstly, it minimizes the possibility of inducing errors, as the separation is very precise and can easily be completed with an additional flat-bed check by using the same material, which is therefore not lost, and the demands on the amount to be analyzed are consequently very reasonable. Another advantage is based on the fact that the column technique gives a good possibility of recovering unusual amino acids or hydrolysis-resistant peptides, which may be of considerable importance in special situations such as in the analysis of complex peptide mixtures. As indicated in Fig. 4, this technique offers the possibility of separating almost all common amino acids in one run, and, under standard conditions, the technique can

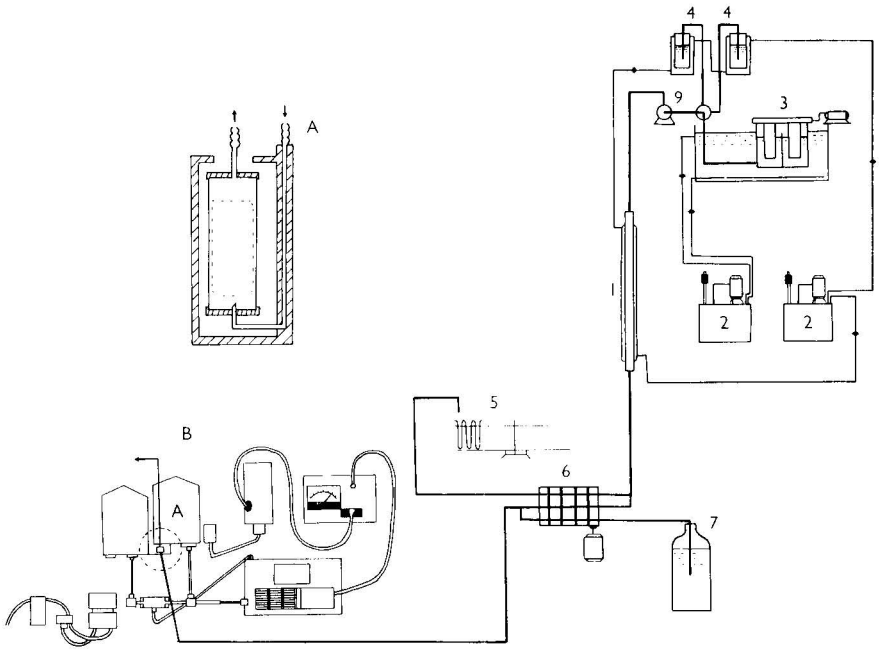


Fig. 3. The overall assembly of the chromatographic equipment. 1 = Separation column; 2 = thermostats; 3 = gradient device and reservoirs (benzene–acetic acid gradient); 4 = reservoirs for benzene–acetic acid (9:1 and 6:4 systems); 5 = fraction collector; 6 = proportionating pump; 7 = acetone reservoir; 8 = Farrand spectrofluorimeter (detail A, flow-through cuvette); 9 = programmed three-way tap.

also be used for quantitative determinations. These advantages are, of course, obtained at the cost of using more complicated equipment and slightly larger samples for analysis (at least twice as much as in the flat-bed technique).

As in every separation of a complex mixture there are pairs of Dns derivatives which are difficult to separate, such as phenylalanine (Phe) and a number of others with high chromatographic mobilities. In order to achieve adequate separations, which may be subjected to quantitation by using the technique common in non-derivatized amino acid analysis, one has to work in the region of *ca.* 2000 theoretical plates. An improved separation has been obtained by introducing a gradient system at the beginning of the chromatographic run. The operating times and solvent

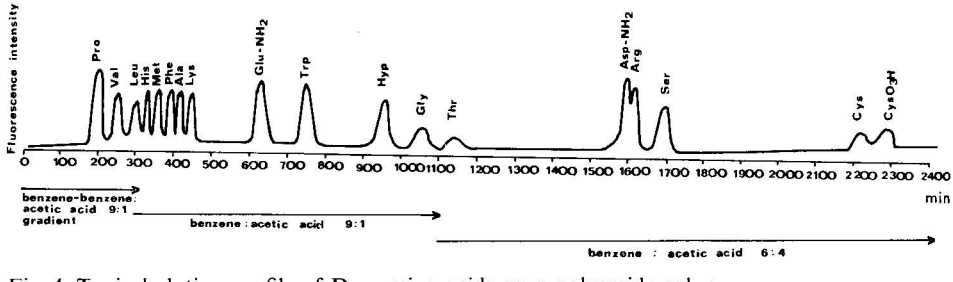


Fig. 4. Typical elution profile of Dns-amino acids on a polyamide column.

TABLE 3

RETENTION VOLUMES AND RELATIVE RETENTION VOLUMES OF Dns-AMINO ACIDS

Amino acid	*Benzene–acetic acid (9:1)*		*Benzene–acetic acid (6:4)*	
	V_e*	V_e/V_{pro}**	V_e	V_e/V_{pro}
Leu	138	1.53	80	1.02
Val	124	1.35	82.5	1.05
His	143	1.64	–	–
Met	152	1.69	81	1.04
Ala	163	1.81	92	1.08
Lys	196	2.18	108	1.38
Gly	765	8.39	117	1.50
Trp	500	5.55	134	1.71
Thr	865	9.60	142	1.82
Ser	2540	28.21	485	6.21
Cys	–	–	980	12.57
Arg	–	–	384	4.92
Hyp	700	7.76	–	–
Pro	90	1	78	–
Phe	155	1.72	85	1.09
Gln	370	4.12	126	1.61
Asn	1350	15.00	370	4.74

* V_e = Elution volume.

** V_e/V_{pro} = Elution volume relative to proline.

systems used are as follows: 0–300 min, gradient of benzene/benzene–acetic acid (9:1), 200 ml of each solvent; 300–1100 min, benzene–acetic acid (9:1); and 1100–2500 min, benzene–acetic acid (6:4). The chromatographic properties of the individual solvent systems used are summarized in Table 3.

Although no precise rules for predicting chromatographic mobility can be formulated, there are general features which, for an unknown derivative, may serve as a guide. An increase in the number of carbon atoms in the amino acid side-chain decreases the retention time. Compared with a straight chain, the difference due to a $-CH_2$ group in a branched side-chain has a much smaller effect in decreasing the retention time. Hydroxylation, however, shifts retention times to much higher values and the differences in a homologous series are increased in hydroxylated amino acids. While the presence of a second amino group causes the amino acid to move with a low retention time, guanidylation considerably retards the chromatographic mobility.

High-speed liquid column chromatography has been introduced by Engelhardt *et al.*[29] for the separation of Dns-amino acids. Silica gel particles of size ranging between 5 and 8 μm were packed into a 50 cm $\times$ 4.2 mm stainless-steel column. The sorbent was dynamically equilibrated with water using water-saturated dichloromethane. In the final stage, the sorbent contained 0.4 g of stationary liquid per gram of silica. Separation was run under 255 atp with a flow-rate of 3.2 ml/min. A very good separation, as exemplified in Fig. 5, was achieved within 22 min with water-saturated dichloromethane as the mobile phase. However, only less polar amino acid derivatives were eluted in this step. Therefore, in the next separation step, a more polar mobile phase was used. If the column is eluted with dichloromethane con-

taining 10% of water, the elution pattern depicted in Fig. 6 is obtained. It appears that with high-speed liquid column chromatography it is possible to resolve in two successive steps most of the common Dns-amino acid derivatives. A continuous gradient elution would presumably give even better results; however, the detection method of choice (not specified clearly in the paper, but presumably with a differential refractometer) in the above procedure was difficult to balance with respect to the baseline shift under gradient elution.

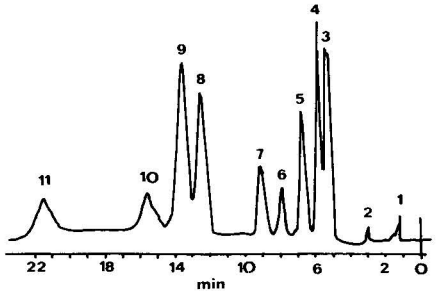


Fig. 5. High-speed separation of Dns-amino acids. (Engelhardt *et al.*[29]). Column: 50 cm (4.2 mm I.D., drilled); spherical silica (Professor Unger, Technische Hochschule, Darmstadt, G.F.R.) dynamically coated with 0.4 g of stationary liquid per gram of silica; particle size 5–8 μm; mobile phase, dichloromethane, water saturated (1500 ppm H_2O) + 1% acetic acid + 1% 2-chloroethanol; pressure, 255 atm; linear velocity, 0.6 cm/sec; flow-rate, 3.2 ml/min. 1 = Inert (k' = 0); 2 = unknown (1.4); 3 = Dns-Ile (2.9); 4 = Dns-Val (3.25); 5 = Dns-Leu (3.9); 6 = Dns-Tyr (4.7); 7 = Dns-Ala (6.5); 8 = Dns-Trp (8.0); 9 = Dns-Gly (8.8); 10 = Dns-His (10.1); 11 = Dns-Lys (14.4).

For laboratories that do not possess the complex high-speed chromatographic equipment, the above technique is still of value as it can also be used with lower pressures according to the size of sorbent particles used.

Very recently another technique, which can be classified as high-speed liquid column chromatography has been published by Yamabe *et al.*[30]. TSK-gel LS-140, a

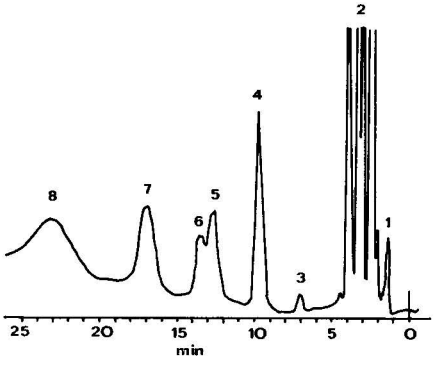


Fig. 6. High-speed separation of Dns-amino acids. (Engelhardt *et al.*[29]). Conditions as for Fig. 5, except 10% 2-chlorethanol in the mobile phase. 1 = Inert; 2 = mixture separated in Fig. 5; 3 = unknown; 4 = Dns-Thr (k' = 5.9); 5 = Dns-Ser (8.0); 6 = Dns-Glu (8.5); 7 = Dns-Asp (11.0); 8 = Dns-Cys (15.5).

macroreticular poly(vinyl acetate) gel (average grain size 10 μm), served as sorbent; *n*-hexane–ethanol–acetic acid–triethylamine (90:10:1:1) was used as mobile phase at a flow-rate of 2.2 ml/min and at a pressure of 83 atm. The column size was 50 cm × 3 mm and running time only 60 min; however, no baseline separations were obtained with a standard mixture of amino acids, as indicated in Fig. 7.

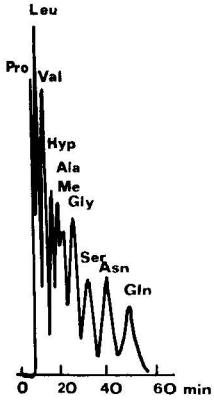


Fig. 7. Chromatogram of Dns-amino acids using the mobile phase *n*-hexane–ethanol–acetic acid–triethylamine (90:10:1:1). Flow-rate, 2.2 ml/min; pressure, 83 kg/cm^2.

3. BANSYL DERIVATIVES

The replacement of the dimethylamino group of Dns-Cl with a di-*n*-butylamino group offers some advantages over Dns derivatives, the resulting compounds being less polar and easily separated in non-polar solvents (Seiler *et al.*[31]). The derivatization is carried out at room temperature in acetone–water (3:1) saturated with sodium carbonate, in a similar way to that with Dns-Cl. Thin layers of silica or polyamide can be used for the separation. With polyamide, benzene–acetic acid (9:1) may serve as mobile phase. Spot positions are obvious from the scheme shown in Fig. 8. The sensitivity range is in the order of tenths of a nanomole, which appears to be another important advantage of these derivatives.

4. DABSYL DERIVATIVES

Recently, a new, coloured type of derivative for the N-terminal labelling, 4-N,N-dimethylaminoazobenzene-4′-sulphonyl (dabsyl) chloride, has been synthesized by causing methyl orange to react with phosphorus pentachloride. The sulphonyl groups react readily with primary and secondary amino groups. According to Chang and Creaser[32], the intense chromophoric dabsyl amino acids formed permit the detection of amino acids as coloured spots in the range 10^{-10}–10^{-11} mole.

The silica gel plates originally suggested for thin-layer separation of these derivatives suffer from diffusion effects, which in some instances may obscure the results. Therefore, in later work performed by Chang and Creaser[32] polyamide sheets were used instead. The whole procedure is carried out on 5 × 5-cm sheets and optimum detection is observed with 10–20 pmole of each amino acid.

The practical procedure for preparing dabsyl derivatives is as follows. The requisite amount of each amino acid (50 nmole) is dissolved in 50 nmole of 0.2*M* $NaHCO_3$ and allowed to react with an equal amount of dabsyl chloride in 50 μl of acetone at 70° for 5–10 min; 10–15 pmole are then applied to the 5 × 5-cm polyamide sheet. It has been stressed that the size of the starting spot should not exceed 1.0–1.3 mm. In general, the solvent systems of Woods and Wang[17] originally proposed for Dns-amino acids are recommended. Thus in the first dimension the plate is developed in water–2-chloromethanol–formic acid (100:60:3.5) while in the second dimension the plate, after being carefully dried, is developed in benzene–acetic acid (6:1). A schematic representation of the separation is presented in Fig. 9. The spots can be intensified by exposure to HCl vapour.

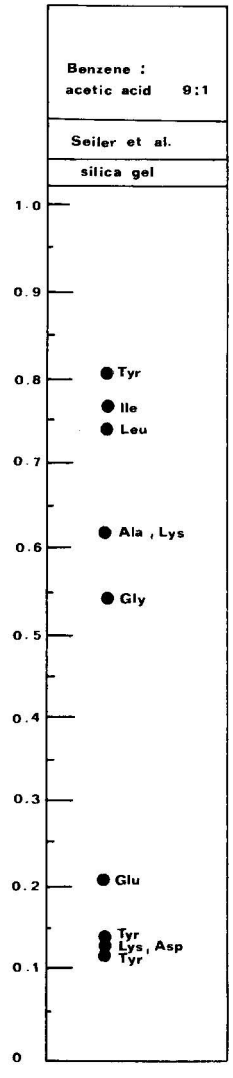


Fig. 8. Schematic representation of the separation of bansyl derivatives.

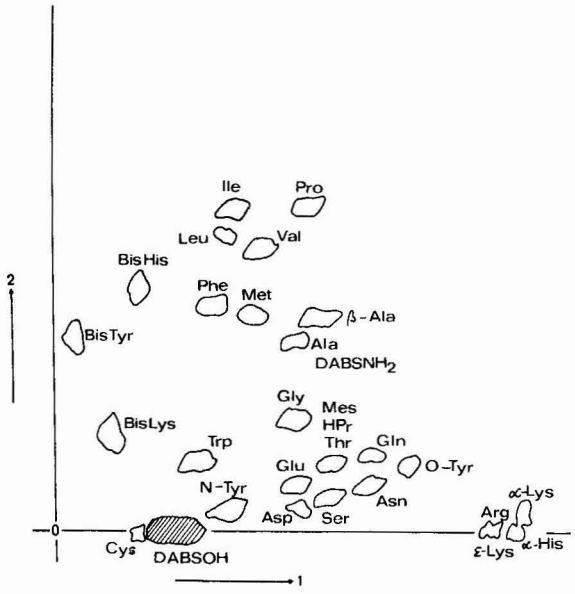


Fig. 9. Schematic representation of the separation of dabsyl derivatives.

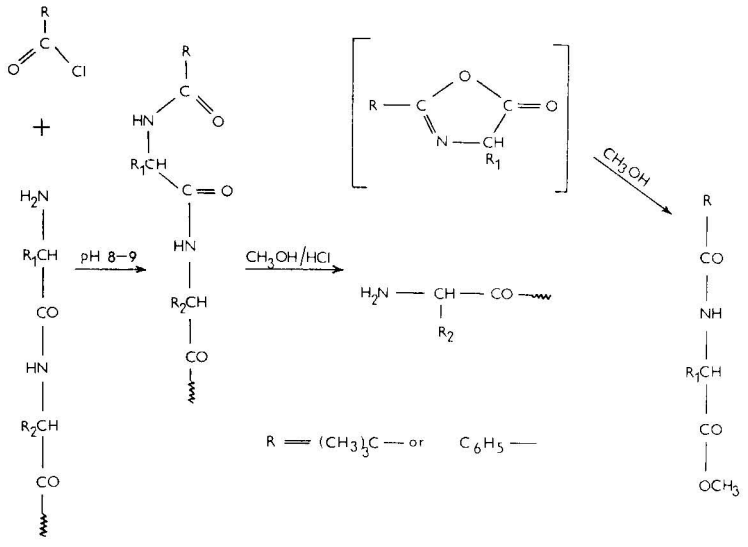
In the above system, several combinations of amino acids are not separated, thus the spot of cysteic acid is fused with that of dabsyl-OH, methionine (Met) sulphone is indistinguishable from hydroxyproline (Hyp) and Thr, and α-Lys derivative and α-His derivative form a combined spot; Arg and Lys are also difficult to separate. The use of other solvent systems that allow the separation of these overlaps was suggested. Thus dabsyl-OH and cysteic acid (corresponding derivative), dabsyl-NH_2 and Ala, methionine sulphone, Hyp and Thr, and α-monohistidine, α-monolysine and Arg, can be separated (always from the last-mentioned amino acid derivative) by developing the plate in water–pyridine–28% ammonia–formic acid (100:20:10:2). Arg can be separated from monosubstituted Lys and His by developing the plate in water–28% ammonia–ethanol (9:1:10). Very recently Creaser[33] used the isothiocyanate derivative of the dabsyl reagent for direct sequencing work.

5. PIVALYL AND BENZOYL DERIVATIVES

(A) Derivatization

Both pivalyl and benzoyl chloride are reagents that are very reactive towards the N-terminal amino group. Also, the introduction of a benzoyl or pivalyl group facilitates the hydrolysis of an N-terminal amino acid; both of these reagents were used for the first time by Cavadore *et al.*[5]. The final identification was achieved by gas chromatography after converting the liberated benzoyl and pivalyl derivatives into their corresponding methyl esters. Schematically, both reactions can be represented

as follows:



In practice, 10–15 μmole of the peptide to be analysed are dissolved in 100 μl of N-ethylmorpholine contained in a vial tube and 20 μl of pivalyl chloride or benzoyl chloride are added. The reaction is allowed to proceed for 30 min at room temperature and the solvent is then evaporated off in a stream of nitrogen. The residue is dissolved in 300 μl of methanol saturated with hydrogen chloride and heated to 70° for 30 min. The acidic methanol is evaporated off and the residue is dissolved in 200 μl of 0.1 *N* HCl and the solution extracted twice with ethyl acetate to give a total of 600 μl. The extract is evaporated to dryness, re-dissolved in methanol and treated with an excess of diazomethane in diethyl ether for several minutes. The reaction mixture is then taken to dryness, dissolved in 5 μl of dichloromethane containing the corresponding internal standard and this solution is directly transferred to the gas chromatograph.

If the molecular weight of the peptide or protein is too large, it has been recommended that about 100 nmole of the substance to be analysed be suspended in an acetone–water mixture (1:1) and only then is the N-ethylmorpholine solution added.

(B) Gas chromatography

For the chromatographic separation of N-pivalylamino acids in the form of their corresponding methyl esters, a 35 m × 0.028 cm capillary column, coated with 5% XE-60 and FFAP (0.5%) in dichloromethane was used. Nitrogen served as carrier gas at a flow-rate of 1 ml/min. Temperature programming and separation of individual amino acid derivatives can be seen in Fig. 10. Pivalylphenylalanine ethyl ester served as internal standard.

An identical column was used by Cavadore *et al.*[5] for the separation of benzoylamino acid methyl esters. The column was coated with 1% FFAP in dichloromethane and nitrogen served as carrier gas at a flow-rate of 1 ml/min. The injector heater in both instances was heated to 250°. Temperature programming of the column

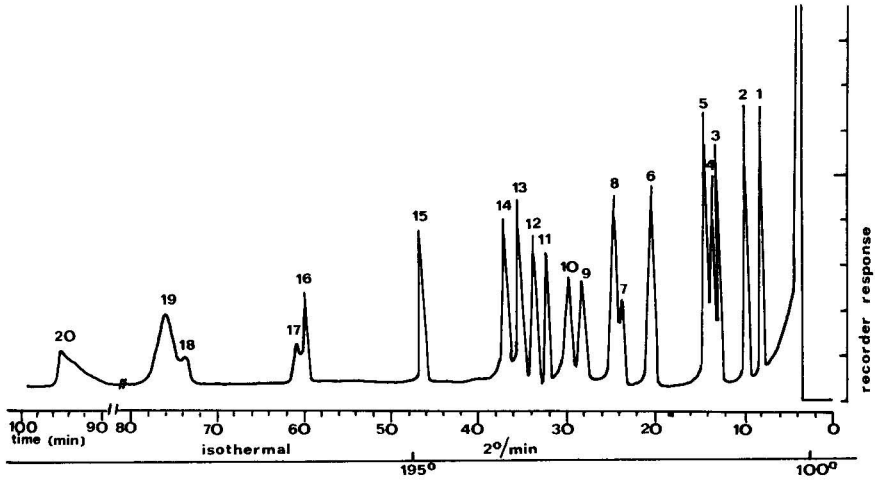


Fig. 10. Gas chromatographic separation of an equimolar mixture of N-pivalylamino acid methy esters (5 nmole) on a glass capillary column (35 m × 0.028 cm I.D.) coated with XE-60 (5%) and FFAP (0.5%) in CH_2Cl_2. Nitrogen carrier gas flow-rate, 1 ml/min.; injector heater at 250°. The internal standard used was pivalylphenylalanine ethyl ester. Peak identification: 1 = Ala; 2 = Val; 3 = Ile; 4 = Gly; 5 = Leu; 6 = Pro; 7 = S-CH_3-Cys; 8 = Asp; 9 = Thr; 10 = Ser; 11 = Met; 12 = Glu; 13 = Phe; 14 = internal standard; 15 = S-CM-Cys; 16 = Tyr; 17 = Asn; 18 = Gln; 19 = Lys; 20 = Trp.

and the quality of separation are shown in Fig. 11; good peaks were obtained after injecting 5 nmole of the derivative into the apparatus.

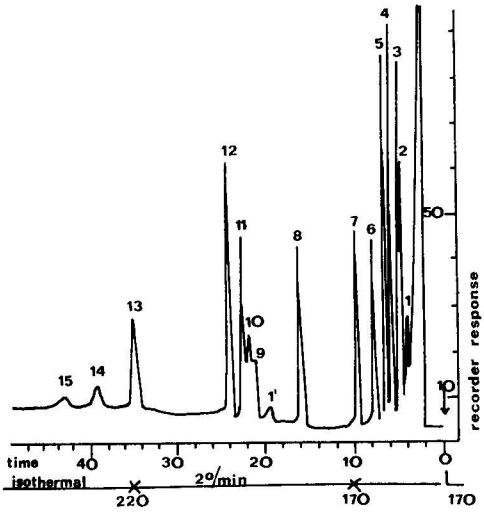


Fig. 11. Gas chromatographic separation of an equimolar mixture of N-benzoylamino acid methyl esters (5 nmole) on a glass capillary column (30 m × 0.028 cm I.D.) coated with FFAP (1%) in CH_2Cl_2. Nitrogen carrier gas flow-rate 1 ml/min; injector heater at 250°. Peak identification: 1, = Ser; 2 = Ala; 3 = Val; 4 = Ile; 5 = Leu; 6 = Gly; 7 = Pro; 8 = Asp; 9 = Thr; 10 = Met; 11 = Glu; 12 = Phe; 13 = Tyr; 14 = Asn; 15 = Gln.

6. PHENYLTHIOHYDANTOINS

(A) Flat-bed separations

For many years thin-layer chromatography of phenylthiohydantoins (PTHs) has been considered a simple and reliable method for the identification of amino acid residues liberated during the individual steps of Edman's degradation procedure[34]. The trends in this field are, like those with the dansylation procedure, directed towards increased sensitivity, which would compensate for stepwise losses and decreased yields recorded during the individual steps of the sequencing process. Recently, chromatography on small-size polyamide sheets has been used for detecting 0.05–0.20 nmole of PTHs. The sheet size used was 5 × 5 cm and detection was carried out by the conventional quenching of a fluorescent indicator added to the layer. Summers *et al.*[35] reported the possibility of detecting as little as 0.3 nmole on 6.3 × 6.3 cm plates using the starch–iodine reaction and Edman[36] himself reported that it was possible to quantitate 0.8 nmole on 20 × 20-cm silica gel plates, and the detection limit in the latter instance was 0.4 nmole.

Generally speaking, small-size plates are currently preferred as the spots, which move a shorter distance, are more compact and smaller amounts can therefore be recovered. On the other hand, Inglis and Nicholls[37], again using 20 × 20 cm plates, succeeded in reliably detecting less than 0.2 nmole and surmised that if smaller plates were used, the sensitivity would accordingly be higher. The method is based on exposing the plate, after careful removal of the mobile phase, to iodine vapour. While Truter[38] does not report this procedure as being particularly suitable for the estimation of PTHs, our own results obtained by the method of Inglis and Nicholls[37] indicated very good applicability of the iodine detection.

It should be stressed that the method of Inglis and Nicholls differs from that involving the iodine–azide reaction, the use of which was not recommended (Feigl[39] and Edman[36]), although it has been used extensively to increase detection limits; the main problem here lies in the fact that the intensity of the spot has a poor correlation with the amount of PTH actually present. This may obviously be a source of serious errors in sequencing. As stressed by Inglis and Nicholls, detection by fluorescence quenching suffers similar problems, while in the iodine reaction these problems are minimized. The reaction with iodine vapour is reversible and non-destructive (Barrett[40]) and therefore offers the possibility of subjecting a particular spot to further investigation.

With regard to other detection methods, that with ninhydrin–collidine mixture in absolute ethanol is still in use (Roseau and Pantel[41]). The chemical reactions involved in this detection were elucidated by Schäfer and Bauer[42]. It has been proved that the red colour obtained after spraying the plate is that of hydrindantin, which is further supported by the results obtained with acetyl-PTHs, as reported by Inglis and Nicholls[43]. These derivatives mostly do not give coloured reaction products, which fact is strongly indicative of the participation of the imino group of the thiohydantoin ring in the detection reaction. The reaction with ninhydrin–collidine in ethanol, although less sensitive by at least by one order of magnitude than the above described procedures, is of considerable diagnostic value as it results in different colours being obtained with different amino acid derivatives, as summarized in Table 4.

TABLE 4

SPECIFIC COLOUR REACTIONS OF PTH-AMINO ACIDS WITH NINHYDRIN–COLLIDINE REAGENT

Amino acid	*Time of heating (min)*	*Colour*
Ser	1	Red-violet
Gly	1.5	Intense orange
Ala	1.5	Red-violet
Methionine sulphone	2	Brown
Cystine	3	Intense pink
Cysteic acid	3	Light pink
Asn	4	Light yellow
Glu	4	Greenish brown
Met	4.5	Brown
Glu	4.5	Dark brown with blue halo
His	4.5	Faint yellow
Asp	4.5	Pink
Arg	4.5	Very faint yellow
Trp	5	Intense yellow
Tyr	5	Light yellow
Thr	5	Light brown
Lys (ε-)	7	Very faint pink
Pro	8	Very faint pink
Phe	9	Very faint yellow
Leu	15	Very faint grey
Ile	15	Very faint grey
Val	—	No colour

With regard to solvent systems used for the TLC separation of PTH-amino acid derivatives, barely any improvements have been made recently and the systems presented earlier[2] may serve to illustrate the older techniques of separation that are still in use and will obviously survive into the future.

The recent paper by Walz and Reuterby[44] reports the use of a flat-bed system that is suitable for routine identification of PTH derivatives originating from the automated Edman procedure. In this paper use is made of the solvent systems employed by Inagami and Murakami[45] and by Jeppson and Sjøquist[46], with pre-coated silica gel plates as sorbent. Some additional information about a particular derivative can be obtained by blowing ammonia vapour over the ninhydrin/collidine developed chromatogram and observing the colour change produced (Table 5).

A frequently occurring problem in the chromatographic separation of PTH-amino acids as well as of methylthiohydantoin(MTH)-amino acids lies in the separation of the arginine, cysteic acid and histidine derivatives. Recently, Kulbe[47] published a thin-layer microseparation procedure for these derivatives; 5 × 5 cm polyamide sheets from two different sources gave satisfactory results when developed with ethyl acetate–*n*-butanol–acetic acid (35:10:1) or with ethyl acetate–*tert.*-butanol–acetic acid in the same proportion. The results are summarized in Table 6. Running time for these separations did not exceed 10 min. In order to improve the contrast under UV light it is recommended that some sheets should be pre-run in the developing solvent system.

In the practical procedure recommended by Kulbe[47], the derivatives of polar

TABLE 5

COLOUR PROPERTIES OF PTH-AMINO ACIDS AFTER NINHYDRIN DETECTION AND SUBSEQUENT EXPOSURE TO AMMONIA VAPOUR

GC = Gas chromatography.

Amino acid	*Colour properties*	*Colour change with NH_3 vapour*
Val	Colourless; use GC	
Pro	UV; colourless	Light blue after heating
Ala	Purple	Deeper color
Gly	Orange	
Ser	UV; purple	
Serine breakdown	Faint orange	Weak red
Asn	Yellow	More intense
δ-Carboxymethylcysteine	UV; purple	
Methionine sulphone	Light tan	
Leu	Colourless; use GC	
Met	Faint tan	
Lys	Very faint pink	Weak blue after heating
Tyr	UV; yellow before spraying	Intense yellow
Thr	Colourless	Light tan
Dehydrothreonine	Colourless*	
Gln	Dark green	Dark blue
Ile	Colourless; use GC	
Phe	UV; colourless	Faint yellow
Trp	UV; yellow before spraying	Deep yellow
Asp	UV; pink	Darker
Glu	Grey	Dark blue

* Characteristic scan at 320 nm.

amino acids, *e.g.*, cysteic acid, histidine and arginine, which remain in the aqueous phase during extraction of methyl- or phenylthiohydantoins, are recovered by lyophilization and re-dissolved in methanol. After application of 1 μl of the solution on the polyamide sheet, chromatographic separation is carried out in the solvent systems mentioned above. The results obtained are believed to be better than those

TABLE 6

SEPARATION OF SOME PTH AND MTH DERIVATIVES ON POLYAMIDE SHEETS WITH SPECIAL REFERENCE TO THE MOBILITY OF ARGININE

Mobile phase	*Amino acid*	*Schleicher-Schuell polyamide*		*Cheng Chin polyamide sheets*	
		MTH	*PTH*	*MTH*	*PTH*
Ethyl acetate–*n*-butanol-acetic acid (35:10:1)	Arginine	0.50	0.50	0.44	0.44
	Cysteic acid	0.50	0.50	0.50	0.50
	Histidine	0.85	0.83	0.82	0.82
Ethyl acetate–*tert.*-butanol–acetic acid (35:30:1)	Arginine	0.29	0.28	0.30	0.30
	Cysteic acid	0.50	0.50	0.50	0.50
	Histidine	0.76	0.78	0.80	0.79

achieved by Rabin and Darbre[48]. As reported by Silver and Hood[49], the use of radioactive phenylisothiocyanate in the automated method parmits the N-terminal sequence analysis of 1.5 nmole of protein. Thin-layer chromatography (two dimensional) is used for the determination of individual split-off amino acids. Separation is carried out on polyamide sheets using 45% aqueous formic acid in the first run followed by development with CCl_4–acetic acid (9:2) in the second run. A sketch drawing of the resulting amino acid map can be seen in Fig. 12. The PTH spots were made visible under UV light, cut out and placed in scintillation vials.

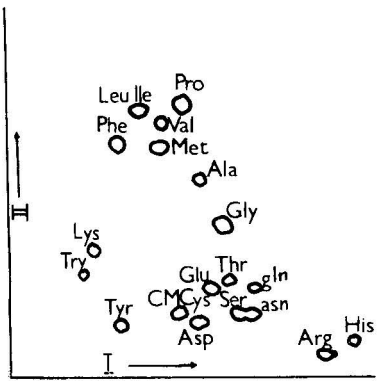


Fig. 12. Separation of PTH-amino acid derivatives by TLC on polyamide sheets. The solvent used in dimension I was 45% formic acid and in dimension II CCl_4–acetic acid (9:2).

(B) Liquid column chromatography and high-speed separations

The application of high-pressure liquid chromatography (HPLC) to PTHs has been introduced only very recently by Frank and Streubert[50] and Matthews *et al.*[51]. Silica columns are used for this purpose and complete separation and quantitation can be achieved in less than 40 min. Good results are also obtained with bonded stationary phases.

In the procedure of Matthews *et al.*[51], a DuPont Model 830 liquid chromatograph was used, with a stainless-steel column (250 × 2.1 mm I.D.) packed with DuPont Zorbax SIL, and operated at 40°. The sample dissolved in methanol (1–10 μl) was injected through a perfluorelastomer septum into the high-pressure line of the column. The system was operated at 1000 p.s.i. which ensured a flow-rate of 0.6 ml/min. Detection was carried out by recording UV absorbance at 254 nm in a 8-μl flow-through cell. Sensitivity of the system ranged between 2 and 5 nmole.

Separations achieved are illustrated in Fig. 13. Elution was carried out with a concave gradient from *n*-hexane–methanol–propanol (3980:9:11) to methanol–propanol (9:11). The shape of the gradient can be described by $C = Kt^5$, where C is the concentration of the second mobile phase in the first, K is a constant and t is the fraction in time of the completed gradient. As indicated in Fig. 13, the general order of elution of individual amino acid derivatives is that of the increasing polarity of the amino acid side-chain.

The main problem with ethyl acetate extractable PTHs in the above separation is the inability to separate PTH-Gly plus PTH-Thr and PTH-Lys plus PTH-Thr. The

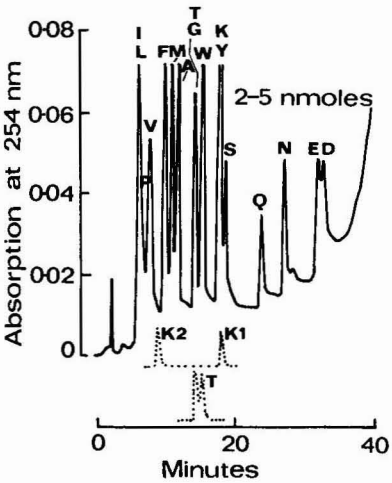


Fig. 13. Separation of PTH-amino acids by HPLC on Zorbax SIL. Elution performed with a concave gradient solvent system from hexane–methanol–propanol (3980:9:11) to methanol–propanol (9:11). Detection is by UV absorption at 254 nm. The PTH-amino acid peaks are identified by the single letter notation for the corresponding amino acids. The elution positions of the two peaks obtained both from lysine (K1 and K2) and from threonine (T) are shown below the main diagram.

authors, however, indicate that in practice this fact should not cause difficulties, because lysine and threonine give double peaks in actual Edman's degradations, which are probably due to the formation of N^{ε}-phenylthiocarbamoyl-N^{α}-PTH-Lys and dehydro-PTH-Thr, in addition to the expected PTH derivatives.

It should be stressed that the above procedure holds for ethyl acetate extractable PTHs. Therefore, problems arise when PTH-Arg or PTH-His have to be assayed. When adjusting the pH of the aqueous phase (which remains after the ethyl acetate extraction) to about 8.0, PTH-His is susceptible to ethyl acetate extraction and can be chromatographed in a linear gradient from hexane–propanol (95:5) to pure propanol. No system was devised by the above authors for the separation of PTH-Arg.

The procedure described by Frank and Streubert[50] involves the use of two independent liquid column systems for the separation of PTHs, those which are more and those less polar. It is claimed that the high-resolution liquid column chromatographic separation is superior to both gas and thin-layer chromatography. The less polar amino acid derivatives, *e.g.* the PTH derivatives of Pro, Ile, Val, Phe, Met, Ala, tryptophan (Trp) and Gly, are eluted with the system dichloromethane–*tert.*-butanol–dimethyl sulphoxide (500:4:0.4). The more hydrophilic group consists of the PTH derivatives of Asp, Asn, glutamine (Gln), Glu, Thr and tyrosine (Tyr) and ε-PTH-Lys, for which series of compounds a mixture of dichloromethane–dimethyl sulphoxide–water (80:15:2) was successfully applied. The separations illustrated in Figs. 14 and 15 were carried out with a Siemens S 200 chromatograph using Merckosorb SI 60 as column packing. Column dimensions were 500 × 3 mm and the column was operated at ambient temperature with a flow-rate of 1.9 ml/min.

Another high-speed procedure that offers excellent results in the separation of

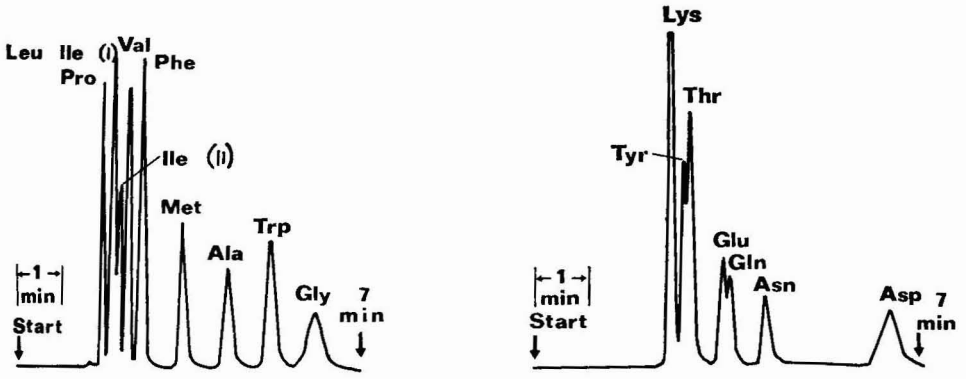


Fig. 14. Separation of PTH-amino acids, "hydrophobic group"; pressure, 250 bar; flow-rate, 1.65 ml/min; mobile phase: CH_2Cl_2–dimethyl sulphoxide–*tert.*-butanol (1000:0.8:8); column: 500-mm tantalum, 3 mm I.D.; pressure, 290 bar; flow-rate, 1.9 ml/min; sensitivity, 1 a.f.s.d.; wavelength, 260 nm; bandwidth, 20 nm; room temperature; sample volume, 10 mm^3; packing: Merckosorb SI 60 (5 μm).

Fig. 15. Separation of PTH-amino acids, "hydrophilic group". Pressure, 250 bar; flow-rate, 0.83 ml/min; mobile phase: organic phase of CH_2Cl_2–dimethyl sulphoxide–H_2O (80:15:2); other conditions as for Fig. 14.

PTH-amino acids is that described by Bollet and Caude[52]: in this instance the separation was carried out with a 25 cm × 2.1 mm column packed with Micropak CN (moderately polar alkyl nitrile phase bonded to silica gel of 10-μm grain size). It is possible to see (Fig. 16) the stepwise elution of individual peaks due to the change in mobile phase composition. Optimum loading capacity in this instance was 3 μl of a sample containing 1–3 mg/ml of the particular amino acid derivative.

Hexane–dichloromethane–isopropanol mixtures of different composition were employed for the preparation of complex gradients used for elution of the individual amino acid derivatives, and the flow-rate varied between 50 and 100 ml/h. The results are summarized in Fig. 17.

(C) Gas chromatography

Gas chromatography has been shown to be a potentially suitable method for the separation of PTHs; however, considerable difficulties are met with when handling some non-volatile derivatives. Trimethylsilyl (TMS) derivatives (Pisano and Bronzert[53] and Harman *et al.*[54]) have proved useful, especially when on-column derivatization has been used. The main disadvantages of the above silylation procedure, as summarized by Inglis *et al.*[55], are as follows: the inability to handle the arginine derivative; widely differing responses in the flame ionization detector; and the limited stability of the derivatives. Recently, Inglis *et al.*[55] and Brian *et al.*[56] reported attempts to overcome these difficulties by acylation. These first indications of the plausibility of the acylation procedure were further developed by Inglis *et al.*[55], who used the acetic anhydride–pyridine (1:1) mixture to acetylate PTHs; usually 100 μl of the reagent were sufficient for a 3-mg sample. The reaction was terminated after a suitable period of time (see Table 7) by shaking the mixture with 1 ml of water.

The actual separation was carried out with a Hewett-Packard Model 7620A

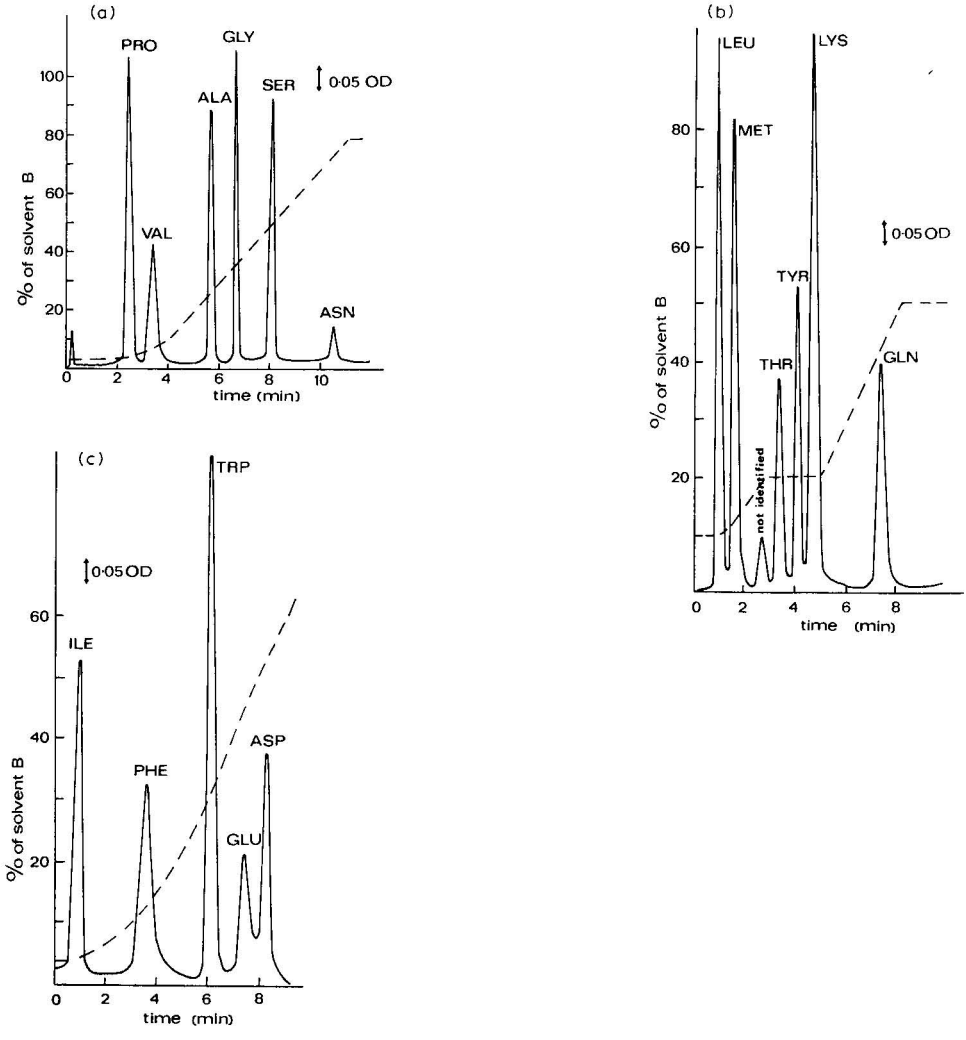


Fig. 16. (a) Separation of six PTH-amino acids (group I): mobile phase (A) *n*-hexane and (B) dichloromethane–isopropanol (8:20); flow-rate, 100 ml/h; overpressure, 90 bar. (b) Separation of six PTH-amino acids (group II): mobile phase (A) *n*-hexane and (B) dichloromethane–isopropanol (1:1); flow-rate, 100 ml/h; overpressure, 100 bar. (c) Separation of five PTH-amino acids (group III): mobile phase (A) *n*-hexane and (B) isopropanol; flow-rate, 100 ml/h; maximum overpressure, 300 bar.

gas chromatograph equipped with a dual flame ionization detector. The injection temperature was 270° and the detection temperature was 10° higher. A 1 m × 2 mm glass column containing 5% Dexsil 300 GC on Chromosorb W (acid washed and silanized) was used at 165° for the first 2 min of running time, programmed at 8°/min to 210° and at 10°/min thereafter up to a final temperature of 290° and maintained at this temperature for 4 min. Helium served as carrier gas at a flow-rate of 25 ml/min. Retention times under these conditions are summarized in Table 7.

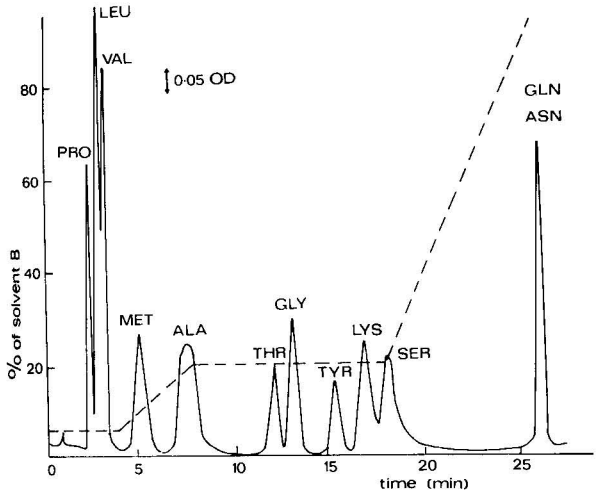


Fig. 17. Separation of PTH-amino acids of all three groups: mobile phase (A) *n*-hexane and (B) dichloromethane–isopropanol (1:1); flow-rate, 50 ml/h; overpressure, 45–100 bar.

In addition, comparison was made with TLC, using the solvent system previously employed by Inglis and Nicholls[37] and silica gel layers.

Clearly, the above procedure is not yet suitable for routine work. The main problems that give rise to obscure results lie not in the separation technique but in the method of derivatization used. The absence of pyridine in the acetylation mixture, although omitted by previous workers, appears to be detrimental, as the acetyl derivative of valine is not formed. Many acetyl derivatives can be prepared by the simultaneous injection of acetic anhydride and the sample directly into the column. In fact, the acetyl derivatives of PTH-Arg and PTH-His can be prepared exclusively by this technique. Other PTHs, such as those of Thr, Ser and carboxymethylcysteine, gave rise to products that moved with retention times that were identical with that of the corresponding glycine derivative. Another series of problems arises from the fact that it is difficult to elute some of the acetylated PTH derivatives from stainless-steel columns. It has also to be stressed that some disagreement exists between different laboratories on this aspect.

Compared with the unacetylated derivatives, the acetyl derivatives exhibit shorter retention times (by about 6%) and about two- to three-fold better response of the flame ionization detector. It has to be pointed out that the acetylation method is obviously not suitable for the separation of acetyl-PTH-Asp, acetyl-PTH-Asn and acetyl-PTH-Gln, which, although acetylated smoothly as indicated by TLC, do not give any response with gas chromatography. Also acetyl-PTH-Leu and acetyl-PTH-Ile do not separate, though this problem had been overcome earlier by Pisano and Bronzert[53] (non-acetylated derivatives).

(D) Conversion of PTHs into parent amino acids

Considerable attention has been paid recently to the hydrolysis and liberation

TABLE 7

ACYLATION CONDITIONS AND CHROMATOGRAPHIC PROPERTIES FOR ACETYLATED PHENYLTHIOHYDANTOINS (INGLIS AND NICHOLLS[37])

Properties of unacetylated PTHs are given in parentheses.

Amino acid	*Reaction time at 20°*	*Mass-spectral analysis*	*GC retention time (min)*		*TLC R_F value*	
Ala	14 h	Monoacetylated	5.04	(5.28)	0.61	(0.40)
Ser	10–40 min	Mono- + diacetylated	5.60		0.51	(0.11)
					0.68*	
Gly	14 h	Monoacetylated	5.64	(5.96)	0.58	(0.35)
S-CM-Cys**	10 min–6 h	Mono- + diacetylated	5.80	Weak response	0.31	(0.16)
					0.50	
Val	14 h	Monoacetylated	6.08	(6.44)	0.65	(0.46)
Pro	—	Not determined	—	(7.20)		(0.55)
Ile	14 h	Monoacetylated	7.20	(7.52)	0.65	(0.50)
Leu	14 h	Monoacetylated	7.20	(7.52)	0.67	(0.52)
Thr	6 h	Diacetylated	7.00		0.49	(0.18)
			7.36***			
Hyp	14 h	Not determined	9.76		0.54***	
			10.28	(10.00)	0.48	(0.28)
Met	14 h	Monoacetylated	9.88	(10.24)	0.63	(0.44)
Glu	6 h	Monoacetylated	10.24		0.36	(0.20)
Phe	14 h	Monoacetylated	10.40	(11.36)	0.66	(0.45)
Tyr	14 h	Diacetylated	13.12	(13.96)	0.62	(0.25)
Lys	14 h	Diacetylated	13.16		0.63	(0.36)
Trp	6 h	Monoacetylated	14.88***			
			17.28	(17.68)	0.52	(0.41)
Asp	6 h	Not determined			0.32	(0.14)
Asn	14 h	Monoacetylated			0.28	(0.08)
Gln	14 h	Monoacetylated			0.27	(0.09)
His	3 h–6 days	Not determined	13.43***, §			
			13.94			(0)
Arg	3 h–6 days	Not determined	12.50§			(0)

* Spot with $R_F = 0.51$ gradually decreases as second spot increases.

** S-CM-Cys = S-carboxymethylcysteine.

*** Major of two peaks.

§ Must be injected in a fresh aliquot of acetic anhydride for "on-column" conversion.

of the free amino acid from the N-terminal PTH derivative. Mondino *et al.*[57] introduced an open flask system using either a nitrogen or argon atmosphere. In practice, 1 μl of the PTH-amino acid derivative solution containing 0.5 μmole of the derivative is placed in a three-necked flask (25 ml), two lateral necks are stoppered and the contents are evaporated to dryness at 40°. The residue is re-dissolved in 5 ml of 0.1 *N* sodium hydroxide (this solution had argon or nitrogen bubbled through it before use). The flask is then placed on a heating mantle and the reaction mixture refluxed while bubbling inert gas through it at a flow-rate of 0.5 ml/min (the bubbling is begun 1 h before applying heat, the mixture being then heated gently for 16 h). The alkaline solution is then neutralized with 2.5 ml of 0.2 *N* hydrochloric acid, taken to dryness at 40° and re-dissolved in 0.2 *N* hydrochloric acid. A 0.4-ml aliquot of this solution is loaded on to the top of the column of a conventional amino acid analyser.

7. METHYLTHIOHYDANTOINS

At present, all types of chromatographic techniques are available for the separation of methylthiohydantoins as their use for sequencing study has spread considerably during the last few years. Methylthiohydantoin (MTH) amino acids can be used in the manual version of the stepwise degradation in the automated procedure or in solid-phase degradation.

(A) Flat-bed separations

Stepanov and Lapuk[58] reported the possibility of separating MTH-amino acids by TLC. However, not all of the common amino acids could be resolved by their technique. Rabin and Darbre[48] applied polyamide-coated sheets and used toluene–*n*-heptane–acetic acid (60:30:20) and 35% acetic acid as mobile phases. R_F values obtained are presented in Table 8. If these solvents are used consecutively, in a two-dimensional arrangement, they provide a complete separation of the 19 common MTH-amino acids. The spots can be made visible in UV light at 254 nm, in which they exhibit a purple fluorescence.

TABLE 8

$R_F \times 100$ VALUES FOR 19 MTH-AMINO ACID DERIVATIVES IDENTIFIED BY TLC ON POLYAMIDE-COATED PLASTIC PLATES

Amino acid	$R_F \times 100$	
	Toluene–n-heptane–acetic acid (60:30:20)	*35% acetic acid*
Ala	67	70
Arg	2	92
Asn	26	82
Asp	19	70
Cys	45	45
Glu	33	70
Gly	54	74
His	8	93
Ile	89	43
Leu	86	43
Lys	22	60
Met	74	52
Phe	80	35
Pro	90	63
Ser	55	61
Thr	57	45
Trp	42	21
Tyr	18	4–
Val	82	57

Very extensive work on the flat-bed separation of MTHs has been carried out recently by Kulbe[59]. Two generally applicable solvent systems were evolved, which allow the separation of 23 MTH-amino acids. The sensitivity of the method lies within the range 0.05–0.2 nmole, provided that fluorescence detection and double-sided

polyamide sheets were used during the separation procedure. Separation was carried out on small-size polyamide sheets (5 × 5 cm) in order to obtain condensed spots (the spot applied should not exceed 1 mm in diameter). Another interesting feature of this method is that the solvents can be applied consecutively in the same direction of development; a conventional two-dimensional arrangement is also possible. Running time is about 30 min. A review of reported R_F data is summarized in Table 9. The most frequently used solvent systems are toluene–*n*-heptane–acetic acid (100:30:15) and 25% aqueous acetic acid. The latter solvent had previously been used for similar purposes (Kulbe[59] and Kulbe and Nogueira-Hattesohl[60,61]). For some pairs that are difficult to separate in the above two solvent systems, 40% aqueous pyridine–acetic acid (9:1) can be used, the separation being carried out on layers that do not contain the fluorescent indicator. For fluorescence detection the indicator is added instead to the mobile phase or, if two subsequent developments are applied, to the mobile phase that is used for the first run; the mobile phase then contains 250 mg of 2-(4-*tert.*-butylphenyl)-5-(4″-biphenylyl-1,3,4-oxadiazole) (butyl-PBD).

The practical procedure, as described by Kulbe[59], is as follows. The polyamide sheet is developed first in the system toluene–*n*-heptane–acetic acid (100:30:15), and allowed to run for 8 min. The sheet is then dried in a stream of cool nitrogen and run

TABLE 9

$R_F \times 100$ VALUES OF 23 MTH-AMINO ACIDS CHROMATOGRAPHED ON MICRO-POLYAMIDE LAYERS IN SOLVENTS I AND II (ONE DIMENSIONAL) AND ALSO BY DOUBLE DEVELOPMENT IN I FOLLOWED BY II IN IDENTICAL DIRECTION

Solvent I: toluene–*n*-heptane–acetic acid (100:30:15); solvent II: 25% aqueous acetic acid.

Amino acid	$R_F \times 100$		
	I	*II*	*II after I*
Ala	66	66	86
S-Aminoethylcysteine	12	32	38
Arg	4	93	93
Asn	23	80	86
Asp	15	65	69
S-CM-Cys*	18	44/53	62
Cysteic acid*	0	23	29/65
Glu	28	63	74
Gln	31	79	84
Gly	59	72	88
His	6	96	100
Ile	83	41	90
Leu	82	41	90
Lys	17	45	57
Met	73	51	85
Methionine sulphone	27	82	81
Phe	77	30	82
Pro	93	60	100
Ser	56	52	74
Thr*	68/49	44	80
Trp	44	15	51
Tyr	14	29	43
Val	78	51	88

* More than one spot observed; the first is the main spot.

in the second direction for 10 min. The spots are made visible by fluorescence quenching and identified according to the position of standards chromatographed on the reverse side of the sheet. For simultaneous identification of multiple samples, as required in automated sequence analysis, the same solvents can be applied consecutively in the same direction. Spot distribution on polyamide sheets resembles that of PTH-amino acids.

Some suggestions concerning the quantitation of MTH-amino acids after TLC separation were reported by Amirkhanyan and Stepanov[62], but the procedures involved are not yet ready for routine application. It is also worth mentioning that contrary to the findings of Rabin and Darbre[48], Kulbe was able to prepare the MTH derivative of glutamine, which was clearly separable from the corresponding derivative of glutamic acid.

(B) Liquid column chromatography and high-speed separations

A glass column, 72 cm × 0.9 cm I.D., packed with Dowex 50-X8 (600 mesh) was used for this type of separation (Stepanov *et al.*[63]). Prior to application to the column the resin was washed stepwise with 80% ethanol, water, 2 *N* NaOH, water and 2 *N* HCl. A final wash with water brought it to a neutral condition. After each run the column could be regenerated by washing it with 250 ml of distilled water and five or six analyses run without repacking the column; 0.02–2.5 μmole of each PTH derivative were applied in order to obtain optimum separation. Elution was carried out first with water (for 35 min), then with a linear water–ethanol gradient, which was prepared by using two 300-ml flasks containing the respective solvents. Separation was carried out at a flow-rate of 60 ml/h, the eluate being monitored spectrophotometrically at 235, 265 and 315 nm.

Analogous results can be obtained with Hitachi spherical resins (2612 and 3105), when pre-treated in a way similar to that described for Dowex 50-X8. Examples of these separations are presented in Fig. 18. As one would expect, the separation with spherical resins yield narrower peaks and offer a clearer separation of some MTH-amino acids, namely Gly, Ala and Gln. With these resins it is also possible to separate *allo* and *threo* forms of Thr and the particular derivative of Ile. Hitachi spherical resins, however, require a lower flow-rate (30 ml/h) and a higher overpres-

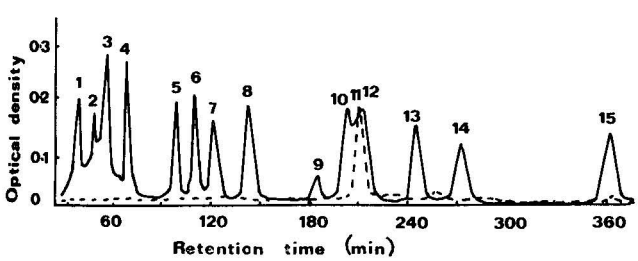


Fig. 18. Separation of MTH-amino acids on spherical analyzing resin. Conditions: resin, Hitachi 2612; elution rate, 60 ml/h; column temperature, 20 ± 0.5°; sample size, 0.2 μmole of each MTH; eluent, water for 0–35 min, then linear gradient water–ethanol (300:300) for 35–340 min. Absorption at 265 nm (———), and at 315 nm (– – – –). Peaks: 1 = Asp, S-CM-Cys; 2 = *threo*-Thr; 3 = Glu, Ser, *allo*-Thr; 4 = Asn; 5 = Gly; 6 = Ala; 7 = Gln; 8 = Val; 9 = *allo*-Ile; 10 = Leu, Met, Ile; 11 = Thr (H_2O); 12 = Tyr; 13 = Pro; 14 = Phe; 15 = Trp.

sure on the column; even so, at the end of the analysis the back-pressure did not exceed 12 atp. It should be mentioned that in these separations the order of the eluted amino acid derivatives did not differ from the sequence of parent amino acids chromatographed under identical conditions.

In contrast to flat-bed techniques, this liquid-column chromatographic separation offers an opportunity to quantitate the results provided that the micromolar coefficients are known. The data for the most common amino acid derivatives are summarized in Table 10.

TABLE 10

MICROMOLAR COEFFICIENTS OF MTH-AMINO ACIDS

Amino acid	n^*	C^{**}	$\pm m^{***}$
Asp	5	14.55	0.29
Ser	7	13.27	0.22
allo-Thr	6	15.28	0.35
Asn	9	16.54	0.10
Glu	7	15.56	0.22
Gly	7	13.22	0.18
Ala	9	14.19	0.20
Gln	6	14.70	0.23
Val	7	15.26	0.34
Met	8	10.98	0.39
Leu	6	14.35	0.22
Tyr	6	14.85	0.33
Thr (dehydrated form)	6	17.44	0.35
Pro	5	12.33	0.20
Phe	9	16.03	0.42
Trp	9	16.68	0.30

* Number of parallel experiments.
** Micromolar coefficients.
*** Mean square deviation.

(C) Gas chromatography

While paper and thin-layer chromatography have been used as almost standard techniques for the separation of MTHs, gas chromatography only recently started to invade this area of separation. Several methods are, however, now available for the separation of this category of amino acid derivatives.

Since the work of Waterfield and Haber[64], further improvements in the separation procedure for MTH-amino acids have been reported by Vance and Feingold[65], Pisano *et al.*[66], Eyem and Sjøquist[67] and Lamkin *et al.*[68]. In the procedure described by Vance and Feingold[65] MTH-amino acids are separated in the form of trimethylsilyl derivatives, a 6 ft. × 1/8 in. I.D. glass column packed with 1% OV-17 and another of the same size packed with 1.5% OV-1 being used for the separations. With the OV-17 column the separation is carried out under the following operating conditions: oven temperature was initially set to 160° and, 3 min after the solvent peak appeared, the temperature was programmed to 260° at a 5°/min gradient and maintained at 260° for an additional 12 min. When the corresponding derivative of tryptophan has emerged from the column the analysis is finished. A typical example of a separation

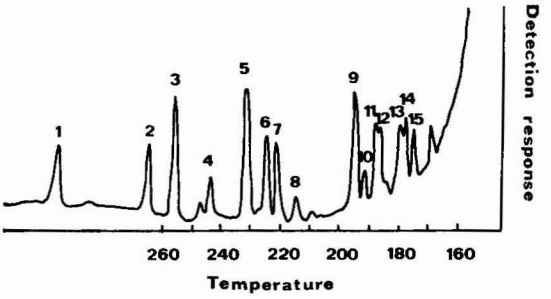


Fig. 19. Gas chromatogram of 18 TMS-MTH-amino acids on an OV-17 column. Oven temperature initially 160°; 3 min after solvent peak appeared, the oven temperature was programmed to 260° for 12 min until TMS-MTH-tryptophan eluted from the column; 2 nmole of each derivative injected, except for the glutamine and lysine derivatives, when 6 nmole was injected. Range setting 10, attenuation 16, resulting in a full-scale deflection of 3×10^{-10} A with a 5-mV recorder. Peaks: 1 = Trp; 2 = Lys; 3 = Tyr; 4 = Gln; 5 = Asn, Phe; 6 = Glu; 7 = Met; 8 = Asp; 9 = Thr, Pro; 10 = Ser; 11 = Leu; 12 = Ile; 13 = S-CM-Cys; 14 = Gly, Val; 15 = Ala.

is presented in Fig. 19. Derivatives of glycine and valine are not resolved under the above conditions and therefore a second run on the OV-1 column has to be carried out. For this purpose, the initial temperature of the oven is 130° and 3 min after the solvent peak has appeared a gradient of 5°/min is set so as to enable the temperature to reach 260° in the final stage of the separation. Amounts ranging between 2 and 6 μmole appear to be the optimum for the analysis. The results of the separation on the OV-1 column is presented in Fig. 20.

The trimethylsilyl derivatives of MTH-amino acids can be prepared by adding N,O-bis(trimethylsilyl)acetamide–acetonitrile mixture (1:3) to the dried MTH derivatives in a small screw-capped vial fitted with a PTFE liner, which is then kept at room temperature for 5 min. The practical procedure used for cleaving the particular N-terminal amino acid from a protein or peptide sample is described by Vance and Feingold[65], and a further description is given by Waterfield and Haber[64].

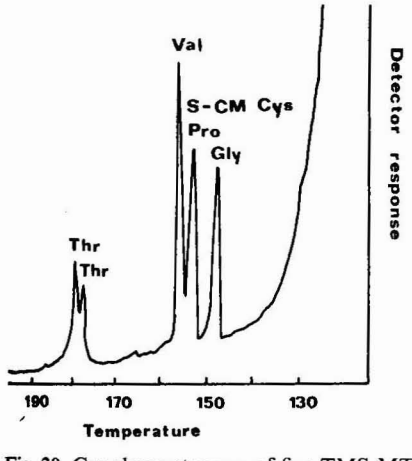


Fig. 20. Gas chromatogram of five TMS-MTH-amino acids on an OV-1 column. Oven temperature initially 130°; 3 min after the solvent peak appeared, the oven temperature was programmed to 260° at 5°/min; 4 nmole of each derivative injected. Range setting 10, attenuation 16.

The application of capillary columns for the separation of silylated MTH-amino acids has been described by Eyem and Sjøquist[67]. In this instance use has been made of a glass column, 4.5 m × 0.13 mm packed with a mixture of OV-101 and OV-225 (95:5). Two different temperature programmes have been developed: in the first, the oven temperature is maintained at 120° for the first 8 min, followed by a temperature gradient of 2.5°/min up to 250°. In the second programme, the initial temperature is 180° for the first 4 min, followed by a 4°/min increase up to the final temperature of 260°. Relative retentions of MTH-amino acids (silylated) are summarized in Table 11. The second programme, outlined above, was designed for the separation of the more labile MTH derivatives, namely the derivatives of Asn, Gln, ornithine (Orn), His and Lys, and for the separation of TMS derivatives of MTH-Glu and Trp.

TABLE 11

RELATIVE RETENTIONS, γ_{is}, OF TMS-MTH-AMINO ACIDS

Amino acid	*1st Programme*		*2nd Programme*	
	γ_{is}*	δ	γ_{is}**	δ
Ala	0.50	0.007		
Gly 1	0.53	0.005		
Val	0.65	0.008		
Pro	0.67	0.005		
Ile 1	0.83	0.007		
Ile 2	0.86	0.001		
Leu	0.88	0.004		
Ser	1.00			
Gly 2	1.11	0.003		
Thr	1.12	0.005		
S-Methylcysteine	1.18	0.007		
Asp	1.39	0.010		
Met	1.43	0.008		
Phe	1.61	0.011		
Glu	1.64	0.011		
Asn	1.71	0.025	0.33	0.005
Gln	1.98	0.020	0.43	0.008
Orn	2.03	0.023	0.46	0.005
His			0.53	0.007
Tyr	2.21	0.023		
Lys	2.27	0.029	0.57	0.003
Trp	2.80	0.031	0.85	0.003

* Relative to phenanthrene.
** Relative to α-cholestane.

The advantage of the procedure described by Eyem and Sjøquist[67] over those reported previously is that by this method it is possible to separate 20 amino acid derivatives in a single run, provided that cysteinyl and arginyl residues have been converted into S-methylcysteinyl and ornithyl residues, respectively. The relative retention in these separations is extremely reliable for identification purposes. Some TMS-MTH derivatives showed a typical pattern (double peaks), which is also of high diagnostic value. Thus isoleucine appears as a double peak, which is ascribed to

the formation of MTH-*allo*-Ile during the silylation procedure. As the mass spectra of the corresponding standard and the newly formed peak were identical, this explanation seems very probable; on the other hand, similar racemization of MTH-Thr was not observed. With the separation of TMS-MTH-Gly, a double peak was also frequently observed, which is reported to be the result of the formation of th bis-TMS derivative.

Precautions have to be taken with the first programme as otherwise the partial decomposition of MTH-Asn, Gln, Orn and Lys and the complete decomposition of His may occur. No problems are reported to arise with the identification of any of the amino acid derivatives except His, because the degree of decomposition is well reproducible. With the second programme higher responses are obtained and also histidine can be identified. Phenanthrene and α-cholestane served as internal standards in the individual programmes devised.

Further investigation of the gas chromatography of MTH-amino acids was devoted to the search for a method that would not require an additional run for the separation of some of the derivatives of commonly occuring amino acids. Although not completely successful, the procedure described by Lamkin and co-workers[68,69] represents at least a further simplification. For this kind of separation two types of column were used: firstly, a column made of borosilicate glass, 165 or 179 cm $\times$ 4 mm I.D. in size, packed with 2% OV-17 on 80–100-mesh Gas-Chrom Q was used. For an additional check, a column packed with OV-25 on 80–100-mesh Supelcoport, which served to confirm the presence of asparagine in the mixture, was also used. In the actual separation procedure, the first column was temperature programmed, starting with 145° for the first 15 min, followed by a temperature gradient of 4°/min. Up to 230° and maintained at this temperature for an additional 12 min. For the identification of arginine the temperature was kept constant at 203° (second column). A typical separation is presented in Fig. 21. Retention indices on SE-30 and OV-17, as reported

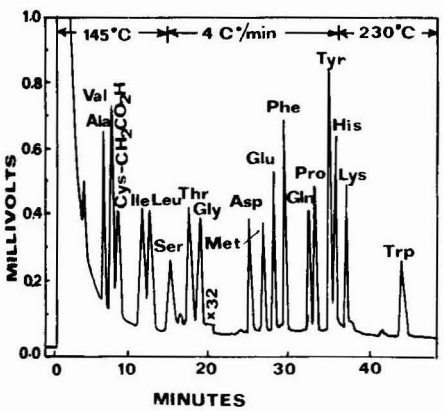


Fig. 21. Gas chromatographic separation of trimethylsilylated methylthiohydantoins (Tracor MT 220). Each peak represents 2.5 nmole of trimethylsilylated methylthiohydantoins, except those for MTH-His and MTH-ε-MTC-Lys, which represent 5 nmole each. Silylation was at 100° for 10.0 min in bis(trimethylsilyl)acetamide–acetonitrile (1:3). Column: borosilicate glass, 165 cm $\times$ 4 mm I.D., containing 2.00% (w/w) OV-17 on 80–100-mesh Gas-Chrom Q. Flow-rate: N_2 at 50 ml/min; H_2 at 50 ml/min; and air at 375 ml/min. Temperature: column programmed as indicated; flash heater, 240°; flame ionization detector, 270°. Attenuation as shown; sensitivity, (attenuation) $\times$ 10^{-11} A/mV.

TABLE 12

RETENTION INDICES OF TRIMETHYLSILYLATED METHYLTHIOHYDANTOINS* OF AMINO ACIDS

Amino acid	*Temperature (°C)***	*Retention index*	
		SE-30	*OV-17*
Ala	140	1491	1724
Val	140	1561	1757
S-CM-Cys	140	1546	1773
Ile	140	1649	1837
Leu	140	1658	1857
Δ-Thr	140	1582	1905
Pro***	140	1543	1905
Gly[c]	140	1611, 1743	1764, 1956
Asp	180	1896	2140
Met	180	1912	2203
Glu	180	2016	2246
Phe	180	2005	2307
Asn	180	2016	2307
Gln	180	2154	2447
Tyr	220	2343	2616
His	220	2298	2645
ε-MTC-lys	220	2326	2721
Trp	220	2631	2926

* Silylated at 60° for 5.0 min in bis(trimethylsilyl)acetamide–acetonitrile (1:3).
** Column temperature at which indices were determined.
*** Not silylated under conditions employed.
[c] Gave two peaks under the silylation conditions employed.

by Lamkin *et al.*[68], are summarized in Table 12. It is interesting to note the dependence of these indices upon the content of the methylphenylpolysiloxane liquid phase employed for the chromatographic separation. This dependence varies for individual amino acid derivatives (as can be seen in Fig. 22), which variation is the cause of changes in the order of elution of individual peaks of amino acids when using different stationary phases.

8. HYDANTOINS DERIVED FROM 2-*p*-ISOTHIOCYANOPHENYLINDONE

These derivatives, the reagent for the preparation of which was synthesized by Ivanov and Mancheva[70], fall into the category of coloured thiohydantoins. For derivatization, the sample (about 1 mg) was dissolved in 0.4 *M* dimethylallylamine buffer (15 ml of pyridine, 10 ml of water and 1.18 ml of dimethylallylamine) and the solution adjusted to pH 9.6 with trifluoroacetic acid. An excess of the reagent (about 4 mg), dissolved in a minimum volume of pyridine, was added and the pH value re-adjusted to 9.6 with trifluoroacetic acid. The coupling reaction was carried out at 40° in an atmosphere of nitrogen, and is usually terminated after 1 h. After completing the reaction, the reaction mixture was extracted four or five times with twice its volume of benzene. Traces of benzene were removed from the mixture with a stream of nitrogen, 0.5 ml of water were added and the aqueous phase taken to dryness by lyophilization. The dry peptide or protein derivative was washed twice with 0.5 ml of

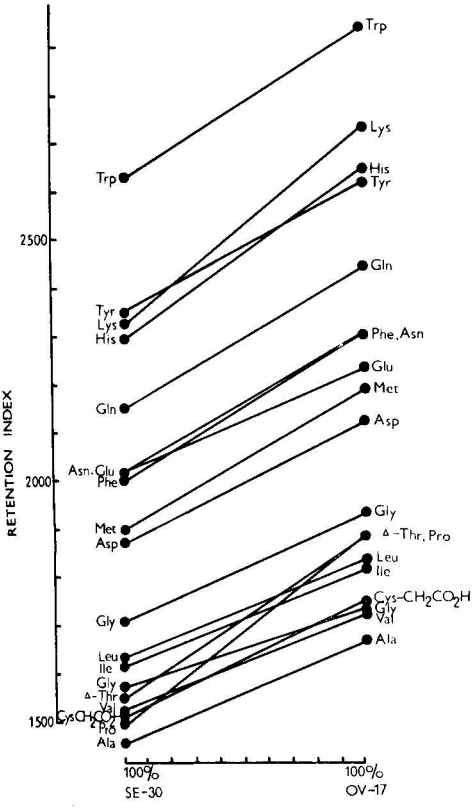


Fig. 22. Retention indices of TMS-MTHs as a function of the phenyl content of the methylphenylpolysiloxane liquid phase employed for chromatographic separation. Silylation was at 60° for 5.0 min in bis(trimethylsilyl)acetamide–acetonitrile (1:3). SE-30 is 100% methyl substituted and thus has a phenyl content of 0%; OV-17 is 50% methyl and 50% phenyl substituted and corresponds to a phenyl content of 50%.

ethyl acetate, traces of the extractant were removed in a desiccator and 50 μl of trifluoroacetic acid were added. The cleavage occured within 15 min at 40° in a nitrogen atmosphere. The resulting thiazolinone was extracted twice, first with 1 ml and then with 1.5 ml of dichloroethane. The combined extracts were taken to dryness in a stream of nitrogen and 100 μl of a mixture of acetic acid–6 *N* HCl (5:1) were added, the conversion being carried out at 80° for 10 min in a nitrogen atmosphere. The reaction mixture was then diluted with water to bring the volume to 0.5 ml and the resulting amino acid derivatives were extracted three times with 1-ml portions of ethyl acetate. The extract was taken to dryness in an atmosphere of nitrogen and traces of solvent were removed *in vacuo* over potassium hydroxide. The sample was then dissolved in 1,2-dichloroethane, acetone or methanol and spotted on the starting line of a thin-layer plate. The conditions for the reaction have been studied extensively by Ivanov and Mancheva, who, for the separation applied thin-layer chromatography on silica gel G layers[70].

The following mobile phases proved suitable for the separation of these amino

acid derivatives[71]: chloroform–methanol (98:2); chloroform–methanol (90:10); *n*-heptane–1,2-dichloroethane–propionic acid (60:20:20); and chloroform–methanol–acetic acid (80:20:2). R_F values obtained are summarized in Fig. 23. As expected,

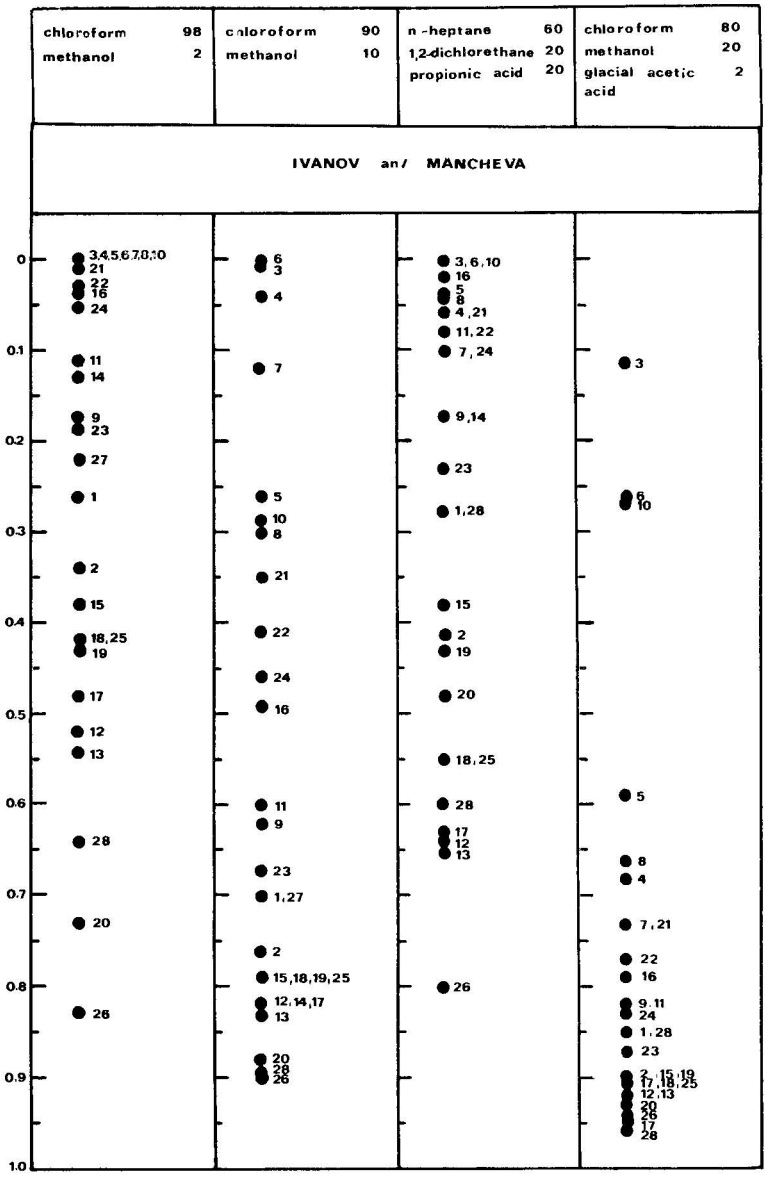


Fig. 23. $R_F \times 100$ values of ITH-amino acids, 2-*p*-isothiocyanophenyl-3-phenylindone and mono- and bisdiphenylindonylthioureas. Peaks: 1 = Ala; 2 = DC-Aminobutyric acid; 3 = Arg; 4 = Asp; 5 = Asn; 6 = $CysO_3H$; 7 = Glu; 8 = Gln; 9 = Gly; 10 = His; 11 = Hyp; 12 = Ile; 13 = Leu; 14 = Lys; 15 = Met; 16 = $MetSO_2$; 17 = NLeu; 18 = NVal; 19 = Phe; 20 = Pro; 21 = Ser; 22 = Thr; 23 = Trp; 24 = Tyr; 25 = Val; 26 = Diphenylindonyl isothiocyanate; 27 = Monodiphenylindonylthiourea; 28 = Bisdiphenylindonylthiourea.

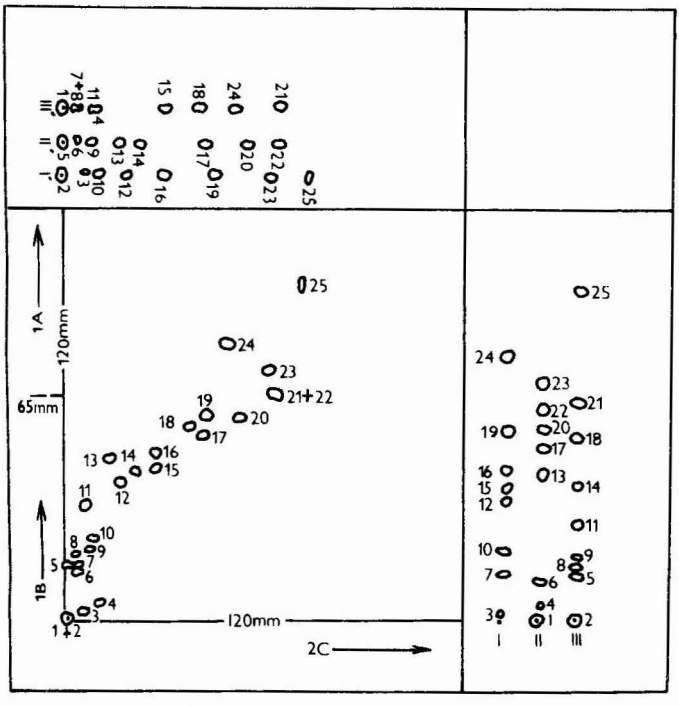


Fig. 24. Two-dimensional separation of ITH-amino acids, diphenylindonyl isothiocyanate and mono- and bisdiphenylindonylthiourea. Sorbent: Kieselgel G; layer thickness, 0.5 mm. First run: solvent system B, chloroform–methanol (90:10), up to 65 mm from the starting line, and in solvent system A, chloroform–methanol (98:2); second run: solvent system C, *n*-heptane–1,2-dichloroethane–propionic acid (60:20:20). Spots: 1 = ITH-cysteic acid; 2 = ITH-Arg; 3 = ITH-Asp; 4 = ITH-Glu; 5 = ITH-His; 6 = ITH-Asn; 7 = ITH-Gln; 8 = ITH-Ser; 9 = ITH-Thr; 10 = ITH-Tyr; 11 = ITH-Hyp; 12 = ITH-Gly; 13 = ITH-Lys; 14 = ITH-Trp; 15 = monodiphenylindonylthiourea; 16 = ITH-Ala; 17 = ITH-α-aminobutyric acid; 18 = ITH-Met; 19 = ITH-Phe; 20 = ITH-Val; 21 = ITH-Leu; 22 = ITH-Ile; 23 = bisdiphenylindonylthiourea; 24 = ITH-Pro; 25 = diphenylindonyl isothiocyanate.

none of the mobile phases specified above is capable of completely separating all of the common amino acid derivatives. Therefore, a special type of two-dimensional chromatography has been applied: the plate is developed in chloroform–methanol (90:10) up to 65 mm from the starting line, and the mobile phase is then changed abruptly to chloroform–methanol (98:2). The development is carried out for an additional 120 mm and the plate is then dried, rotated through 90° and developed in the second dimension with *n*-heptane–1,2-dichloroethane–propionic acid (60:20:20). The result of such a separation is presented in Fig. 24. No detection is needed in this instance because 10^{-9} mole of amino acid derivatives is already visible as a yellow-orange spot.

9. 4-N,N-DIMETHYLAMINOAZOBENZENE-4′-THIOHYDANTOINS (ISOTHIOCYANATE DERIVATIVE OF THE DABSYL REAGENT)

4-N,N-Dimethylaminoazobenzene-4′-isothiocyanate has been synthetised by

TABLE 13

BASIC DATA RELATED TO THE DIFFERENT IDENTIFICATION PROCEDURES IN PROTEIN SEQUENCING

GC = Gas chromatography; LC = liquid chromatography; TLC = thin-layer chromatography.

Type of derivative	*Separation technique used*	*Sensitivity range (method used for quantitation)*	*Unseparated double peaks or amino acid derivatives causing problems in identification*	*Notes*
Dns	TLC (polyamide)	5–20 pmole (photocopying densitometry)	Complete separation with three runs except Dns-Cys	
^{14}C-Dns	TLC (polyamide)	100–500 pmole (radio-activity counting)	Complete separation with three runs except Dns-Cys	
Dns	LC, high speed (silica)	Nanogram (UV recording)	Two runs with differently polar mobile phases necessary	
Dns	LC, classical (polyamide)	Nanogram (luminescence measurement)		
Bansyl	TLC (polyamide)	0.1 nmole (luminescence measurement)	Some double peaks may cause problems (not yet clear)	
Dabsyl	TLC (polyamide)	10–20 pmole (coloured and fluorescent spots)	Complete separations require more than one two-dimensional separation	Sensitivity can be increased by exposing the plate to HCl vapour
Pivalyl derivatives (as methyl esters)	GC, 5% XE and 0.5% FFAP, temperature programming	≈ 5 nmole	Some double peaks may cause problems (not yet clear)	
Benzoyl derivatives (as methyl esters)	GC, 1% FFAP, temperature programming	~5 nmole		
PTH	TLC (polyamide)	0.05–0.2 nmole (5 × 5-cm sheets) 0.8 nmole (20 × 20 cm sheets) (fluorescence quenching)		
PTH	TLC (polyamide)	≈10 nmole (ninhydrin–collidine)		Different colours of different amino acids

PTH	**LC, high speed (Zorbax SIL, Merckosorb SI®, Micropak CN)**	**2–5 μmole**	**Depends on the type of column packing and mobile phase used. Hydrophilic derivatives frequently cause problems in separation**	
Acetyl-PTH	GC, 5% Dexsil on 300 GC Chromosorb W	Data not available	S-CM-Cys, Ser, Thr and Gly, and Leu and Ile, give a single peak	
PTH, after conversion into parent amino acids	Conventional amino acid analysis	>0.5 μmole		
MTH	TLC (polyamide)	0.05–0.2 nmole (5 × 5-cm sheets, fluorescence quenching)	Leu and Ile are difficult to separate. The derivative of glutamine is not easy to prepare	Spot applied should not exceed 1 mm in diameter
MTH	LC, classical (Dowex 50-X8) LC, high resolution (Hitachi spherical resin)	0.02–2.5 μmole (spectrophotometric detection at 235, 265 and 315 nm)		Order of elution of MTH derivatives is the same as that of the free amino acids
MTH (trimethylsilylated)	GC, 1–2% OV-17, then 1.5% OV-1; alternatively, OV-101, OV-225 or OV-25 on Supelcoport. Temperature programming	Data not available		Some trimethylsilylated derivatives of MTH-amino acids give double peaks; 2% OV-17 on Gas-Chrom Q allows the complete separation in a single run
Hydantoins derived from 2-*p*-isothiocyanophenyl-indone	TLC (silica gel G)	10^{-9} mole (orange-yellow spots)		
Isothiocyanate derivative of the dabsyl reagent	TLC (polyamide)	Picomole level		Leu and Ile do not react with the reagent. The rate of the colour change from purple to blue is of diagnostic value

Creaser[33] in order to improve the sensitivity and ease of determination of amino acids liberated during sequence analysis. Derivatives of all amino acids are readily prepared with this reagent (except Leu and Ile, which do not react) and are susceptible to complete two-dimensional separation on thin-layer polyamide sheets. The sensitive azo group permits the detection of the dabsyl isothiocyanate derivatives down to the picomole level as red spots directly on the sheet. Two to three subsequent amino acids at the N-end can be identified by this reagent. The colour change of the spots from purple to blue and red after being exposed to HCl vapour, which corresponds to the conversion of isothiocyanates into the corresponding thiocarbamyl-amino acids, is an additional feature of these derivatives that is of high diagnostic value.

10. CONCLUSIONS

In our opinion the separation techniques presented above characterise the new trends that have been applied for the separation and identification of N-terminal amino acids during sequence analysis. So far, no basically new concepts have appeared in the available literature, the procedures presented performing, in principle, only more precise variations of techniques that have been known for at least 5 years (for a summary, see Table 13). However, interest is such that publication of new procedures for sequence analysis of proteins and peptides surely cannot long be delayed. During a recent discussion at the Meeting on Protein Structure and Evolution, S. W. Fox suggested the possibility of making use of the different rates of splitting of peptide bonds in which different amino acids participate, and subjecting these data to computer analysis, which would result in information being obtained on the most probable sequence of the protein studied. Currently, the techniques are limited to rather short sequences. Another idea concerning sequence analysis relates to mass spectrometry. If the first of these trends materializes, the chromatographic techniques will shift towards more precise quantitation of complex peptide mixtures, with high preference for automated systems. In the second instance, the basic problems will require the use of gas chromatography for their solution. It may be of interest to review such techniques sometime in the future, but at present they lie outside the scope of the this review.

11. NOTE

During the preparation of this manuscript we became aware of the fact that a new technique had been developed for the flat-bed separation of mansyl (N-methyl-2-aniline-6-naphthalenesulphonyl) derivatives of amino acids[72].

12. ACKNOWLEDGEMENTS

The author feels deeply obliged to numerous prominent scientists in this field, namely Drs. Bollet, Caude, Creaser and Kulbe, for making available unpublished results for the purpose of this review. Thanks are also due to Mrs. M. Ciprová and J. Krausová for their work on the final preparation of the manuscript.

13. SUMMARY

New trends in the chromatographic analysis of N-terminal amino acids have been reviewed. It appears that while attempts have been made to achieve more precise separations of PTH and Dns derivatives, separations that could be subjected to quantitative evaluation are preferred. Other trends favour the application of different fluorescent derivatives, such as the dabsyl or bansyl derivatives, which could be used for sequencing of very small amounts of proteins. Miniaturization of scale can be discerned as the second major trend in this area of chromatographic techniques.

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CHREV. 95

THIN-LAYER CHROMATOGRAPHY OF CARBOHYDRATES

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CONTENTS

1. INTRODUCTION

Not long ago Scherz *et al.*[1], in a clear and concise review, discussed the thin-layer chromatography (TLC) of sugars and related compounds which had been developed before 1967. Our review is intended as a continuation of the work of Scherz *et al.*, and deals with the techniques and the results which have been obtained since 1967. However, in some cases, we have re-examined, more critically and in greater detail, those results and techniques which we feel were dealt with too summarily and, therefore, incompletely in the previous review.

2 GENERAL REMARKS ON THE CHROMATOGRAPHIC MODEL

In the chromatography of sugars on layers of cellulose, it is probable that the separation mechanism is based essentially on the liquid–liquid partition principle, according to a model in which the distribution of each sugar between the mobile phase and the "water–cellulose complex"[2,3] or the "liquid gel"[4,5] is expressed quantitatively by the partition coefficient. The relative ease with which the carbohydrates can enter the structure of the complex between water and the solid support reflects the differences in molecular volume and in steric configuration between the various

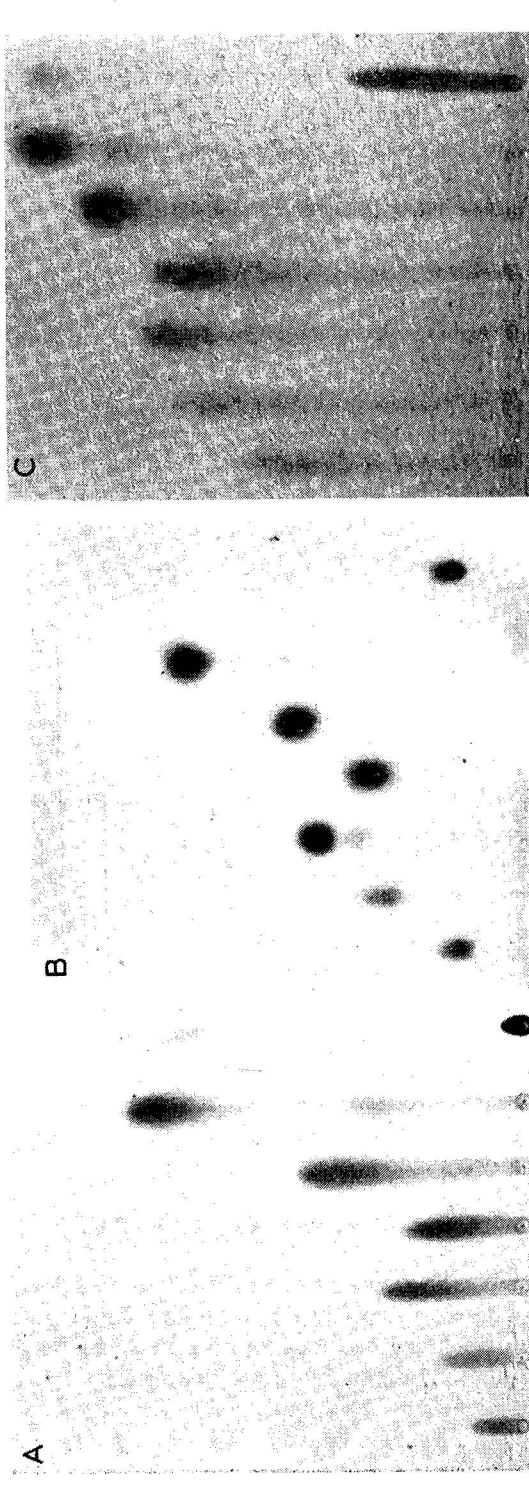


Fig. 1. Chromatography of galactose, glucose, mannose, fructose, ribose, rhamnose and sucrose (from left to right) on silica gel (Merck) impregnated with 0.15 *M* NaH_2PO_4. Solvent systems: A = *n*-butanol–acetone (0.9:1.1); B = *n*-butanol–acetone–water (4:5:1); C = *n*-butanol–methanol–acetone (2:2:1). Solvents B and C are of equivalent polarity. From unpublished results of Lato *et al.*[27].

compounds, and has a fundamental rôle in the separation, together with the differences in solubility of the sugars in a given elution system.

On layers of silica, the partition processes seem to be replaced, to some extent, by adsorption phenomena, so that the chromatography of carbohydrates on this material is clearly influenced by the concentration and type of inorganic salts present in the silica matrix. In this case, the separation is determined not only by the partition coefficient but, also, by the selectivity of the solid matrix.

3. SOLVENTS

The elution systems in the chromatographic separation of sugars are usually organic solvents of binary or ternary composition. Water is an indispensable component, since water-free solvents, or solvents having low water contents, give somewhat diffused spots which compromise the separation (Fig. 1). The content of water varies from 10 to 20% in the solvents for use on cellulose or silica gel, the optimum being 15%. Kieselguhr systems require less than 10% of water.

The binary, water–active solvent, mixtures are efficient eluents, especially for use on layers of silica gel. An active solvent is one which is sufficiently polar to be water-soluble and, at the same time, capable of interacting, through weak solvation bonds, with solutes. Systems such as 2-propanol–water (17:3) and acetone–water (9:1) are among those most frequently used in the analysis of monosaccharides. The ternary systems are made up of water–active solvent–diluting solvent mixtures. The diluting solvent is usually a, or a mixture of two, water-insoluble, or almost insoluble, organic solvent. It influences the flow velocity of the eluting band, whilst the resolving power of the system depends largely on the water–active solvent ratio, which varies from 1:6 to 1:2. For good reproducibility of the results, the ternary system must remain monophasic for the entire chromatographic process. For this purpose, a small quantity of a polar solvent or an organic acid may be added.

The addition of an organic acid to the elution system, other than as a solving agent, has a particular significance in two-dimensional chromatography of carbohydrates, which will be explained later. The best elution systems found in the literature, together with comments, are given in Table 1.

4. SORBENTS

The materials most often used as solid supports in the TLC of carbohydrates are silica, cellulose and Kieselguhr. Some workers have experimented with the use of other adsorbents such as gypsum[6], polyamide[7], aluminium oxide[8] and polycarbonate[9]. The choice of support must be made according to certain criteria: (a) the chromatoplate capacity; (b) the possibility of using at least one of the reagents for the detection of the sugars, and of obtaining a high degree of sensitivity on a clear background; (c) the possibility of resolving a sufficiently high number of carbohydrates.

A. Silica gel

Silica does not give a satisfactory separation of sugars unless it is impregnated with inorganic salts such as bisulphite[10], boric acid[11-13], tetraborate, mono- and dibasic phosphate[14-16] and sodium acetate[13,16], which are capable of interacting with the

TABLE I

SOLVENT SYSTEMS FOR THE TLC OF CARBOHYDRATES AND THEIR DERIVATIVES

Solvent system	*Comments*	*Reference*
To be used on cellulose		
1 Ethyl acetate–pyridine–water (2:1:2)	Galactose, glucose, mannose, fucose and rhamnose are well separated in 3 h	35
2 Formic acid–ethyl methyl ketone–*tert.*-butanol–water (3:6:8:3)	D-Arabinose shows different R_F value from L-arabinose	36
3 Ethyl acetate–pyridine–acetic acid–water (5:5:1:3)	Differentiation of β-methylglucoside from its α-enantiomer, and of α-methylgalactopyranoside from its β-methylgalactofuranoside	37
4 *n*-Butanol–acetic acid–water (3:1:1)		
5 Ethyl acetate–pyridine–water (20:7:5)	Quantitative determination of mono- and oligo-saccharides	39
6 *n*-Butanol–pyridine–0.1 *N* HCl (5:3:2)	Separation of glucosamine and galactosamine together with some hexoses and pentoses	40
7 *n*-Butanol–ethanol–water (3:2:2)	Analysis of oligosaccharides (DP 2–8)	
8 *n*-Butanol–ethanol–water (1:1:1)	Analysis of oligosaccharides analysis (DP 8–14)	42
9 *n*-Butanol–acetic acid–water (4:1:2)	Analysis of ionic charged oligosaccharides	
To be used on silica gel		
10 Acetone–*n*-butanol–water (5:4:1)	Chromatoplates impregnated with inorganic phosphate salts (Soerensen buffer, pH 8)	14
11 Dioxane–*n*-butanol–water (5:4:1)		
12 Ethyl acetate–acetic acid–methanol–water (6:1.5:1.5:1)	Bisulphite impregnation; fair separation of some mono- and di-saccharides	10
13 *n*-Propanol–water (8.5:1.5)		
14 2-Propanol–ethyl acetate–water (7:1:2)		
15 *n*-Butanol–ethanol–water (2:1:1)	Various inorganic salts are used as impregnants; fair separation of some uronic acids	17
16 *n*-Butanol–pyridine–water (8:4:3)		
17 *n*-Butanol–ethanol–0.1 *M* H_3PO_4 (1:10:5)		
18 *n*-Butanol–ethanol–0.1 *M* HCl (1:10:5)		78
19 Ethyl methyl ketone–acetic acid–methanol (3:1:1)		
20 Acetone–water (9:1)	Designed for the resolution of the most common mono- and di-saccharides; impregnation with boric acid and monobasic phosphate	16, 20, 78
21 2-Propanol–water (4:1)		
22 Acetone–water–chloroform–methanol (8:0.5:1:1)		16, 20, 27
23 Ethyl acetate–2-propanol–acetic acid–water (10:6:3.5:3)		27
24 Ethyl acetate–2-propanol–water (2:1:1)	Suitable for the analysis of oligosaccharides on silica gel impregnated with tetraborate, tungstate or molybdate	19, 20, 30, 31

25 ***n*-Butanol–2-propanol–water (3:5:2)**	**0.2 *M* NaH_2PO_4 as impregnant; glucose, galactose, and mannose are separated from each other and from some disaccharides**	**16**
26 *n*-Butanol–ethyl acetate–2-propanol–water (3 5:10:6:3)	Boric acid impregnation; common aldo- and keto-hexoses are well separated (see Table 5)	20
27 Acetone–*n*-butanol–water (7:1.5:1.5) 28 Acetone–*n*-butanol–water (8:1:1) 29 Acetone–*n*-butanol–acetic acid–water (8:0.5:0.5:1) 30 *n*-Butanol–acetone–formic acid–water (60:17:8:15)	Separation of common hexoses, pentoses and deoxy-sugars	27
31 2-Propanol–acetone–1 *M* lactic acid (2:2:1) 32 2-Propanol–acetone–0.1 *M* lactic acid (2:2:1)	Designed for the separation of oligosaccharides (DP 2–6)	53, 56
33 *n*-Propanol–water (7:1) 34 *n*-Propanol–water (7:1.5) 35 *n*-Propanol–ethyl acetate–water (5:1:4) 36 Methyl acetate–2-propanol–water (18:1:1)	Hexosamine separation on unimpregnated silica gel	49
37 2-Propanol–ethyl acetate–water (1:1:2) 38 2-Propanol–ethyl acetate–water (6:1:3)	For the analyses of cello-oligosaccharides	55
39 *n*-Propanol–water (7:3) 40 *n*-Propanol–water–ammonia (7:3:0.1) 41 *tert.*-Butanol–water (2:1) 42 *tert.*-Butanol–water–ammonia (70:35:1) 43 *n*-Butanol–ethanol–water (5:7:4) 44 *n*-Butanol–ethanol–water–ammonia (50:70:40:1) 45 Isopentyl alcohol–ethanol–water (5:6:3) 46 Isopentyl alcohol–ethanol–water–ammonia (50:60:30:1)	Prepared for the analysis of chitin-oligosaccharides	54 54 54 54 54 54 54 54
To be used on Kieselguhr 47 Ethyl acetate–2-propanol–water (4:1:0.5)	Sodium acetate impregnation; quantitative determination of some pentoses of biological interest	34
48 Ethyl acetate–methanol–*n*-butanol–water (16:3:3:2) 49 Ethyl acetate–methanol–propanol–*n*-butanol–water (8:1:1:1:1) 50 Ethyl acetate–methanol–*n*-butanol–water (16:3:3:1)	Suitable for the analysis of sugars of clinical interest	57
51 *n*-Butanol–ethyl acetate–2-propanol–water (20:10:7:3)	Fair separations of hexoses, pentoses and deoxy-sugars	27
52 *n*-Butanol–pyridine–water (45:33:22) 53 *n*-Butanol–pyridine–water (20:19:11)	Separation of malto-oligosaccharides	58

carbohydrates by reasonably well-known mechanisms. In this way, according to the type of impregnating salt and the solvent system used, a wide range of R_F values is obtained on the chromatoplate, and the resolution of the more common sugars becomes possible.

Ovodov *et al.*[17] systematically analyzed the effects of the type of impregnating salt and its concentration on the chromatographic behaviour of certain carbohydrates by using a number of solvents. Their studies showed that a satisfactory separation can be obtained on silica gel impregnated with phosphates. For monosaccharides and uronic acids, the best phosphate concentrations are between 0.2 and 0.3 *M*, and for oligosaccharides between 0.05 and 0.1 *M*. Another important influence of phosphate salts on silica gel is that they cause it to be sufficiently insensitive to the negative interferences of inorganic salt impurities[14,17] and non-saccharide organic compounds such as urea, amino acids, carboxylic acids, etc.[18].

Boric acid and tetraborate, known for their ability to form unstable ionic complexes with carbohydrates, were not among the many impregnants examined by Ovodov *et al.* Earlier, neither Pastuska[11] nor Prey *et al.*[12] obtained satisfactory results with these impregnants, perhaps because of the inadequacy of the acid elution systems used. In fact, as we have pointed out[18,19], the acidity of the solvent system greatly reduces the impregnant effect in the borate chromatoplates. Lato *et al.*[20] carried out extensive studies on the chromatographic possibilities of silica gel impregnated with boric acid. Of the 42 solvent systems examined by these workers, none gave results as good as those obtained with other systems. The resolutions were such that a limited number of carbohydrates could be analyzed by a normal one-dimensional technique. Although, by using larger chromatoplates (20 × 35 cm), it was possible to increase the number of resolvable carbohydrates, the long development time of 12 h proved to be a limiting factor.

Boric acid and tetraborate, when used as impregnants, are the only compounds whose mechanism of action on sugars is well understood. Their capacity to form anionic complexes with polyhydroxy compounds in general, and carbohydrates in particular, has been widely exploited in ion-exchange chromatography[21–26]. A similar mechanism certainly operates in the TLC of sugars in the presence of borate ions. In fact, an examination of Table 2 shows that the chromatographic behaviour of sugars on a layer of non-impregnated silica appears to be compatible with the degree of solubility, and the molecular weight, of the substances examined. When borate ions are present on the silica layer, this behaviour is substantially altered. This suggests that the selectivity of the sorbent (on which the borate ions, insoluble in the mobile phase, develop a primary action in the resolution of the sugars by a complexing–decomplexing mechanism) is a major factor in the chromatographic process[18].

When the borate impregnant is replaced by dihydroxyphenylborane which is soluble in the mobile phase, the selectivity of the solvent assumes a primary rôle in the resolution of the sugars (Table 3). In a two-dimensional technique it is, therefore, important to couple a layer of non-impregnated sorbent (or one impregnated with acetate, phosphates or other salts), in which the separation model is that of partition chromatography, to a borate-impregnated sorbent layer. Following this concept, Mezzetti *et al.*[19] applied the coupled-layer technique of Berger and co-workers[29,30] to the two-dimensional separation of sugars and obtained resolutions of mixtures of monosaccharides, their derivatives and oligosaccharides (Fig. 2).

TABLE 2

R_F VALUES OF SUGARS AND DERIVATIVES, SEPARATED ON (A) UNIMPREGNATED AND (B) 0.05 *M* $Na_2B_4O_7$-IMPREGNATED SILICA GEL (POLYGRAM)

Solvent system: ethyl acetate–2-propanol–water (6:3:1) (ref. 27).

Sugar	R_F	
	A	*B*
α-Methylxyloside	0.68	0.59
2-Deoxyribose	0.65	0.52
Xylose	0.63	0.19
Arabinose	0.54	0.21
Glucose	0.48	0.22
Galactose	0.40	0.15
Levulose	0.51	0.08
Sucrose	0.34	0.20
Melibiose	0.18	0.07
Lactose	0.18	0.08
Melezitose	0.22	0.09
Raffinose	0.13	0.04

TABLE 3

R_F VALUES[28] OF CARBOHYDRATES AND RELATED COMPOUNDS ON WHATMAN NO. 1 PAPER

Solvent systems: A = ethyl acetate–acetic acid–water (9:2:2); B = a 0.55% solution of dihydroxyphenylborane in A.

Compound	R_F	
	A	*B*
Erythrose	0.31	0.84
Erythritol	0.23	0.31
Arabinose	0.12	0.11
Arabitol	0.14	0.50
1-Deoxyarabitol	0.45	0.71
5-Deoxyarabitol	0.46	0.85
Ribose	0.25	0.50
Ribitol	0.14	0.48
Xylose	0.15	0.15
Xylitol	0.14	0.45
Galactose	0.06	0.08
Galactitol	0.07	0.47
Glucose	0.08	0.08
Glucitol	0.08	0.45
Levulose	0.11	0.12
Sorbose	0.10	0.16

The selectivity of the sorbent due to the presence of borate ions may be lost when acid, generally organic, is added to the elution system[19]. This suggests that the sugar–borate complexes are less stable in an acidic environment, and that the complex-formation equilibrium has little influence on the chromatographic separation. However, Bourne *et al.*[28] propose that in acid solution the reaction stops at the stage

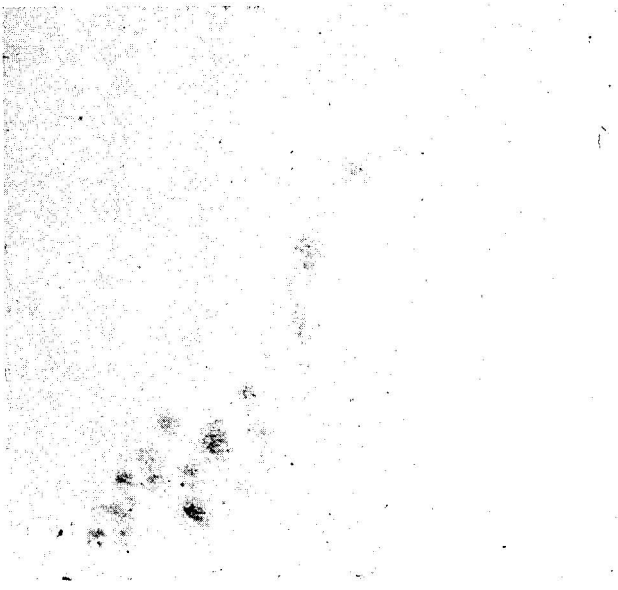
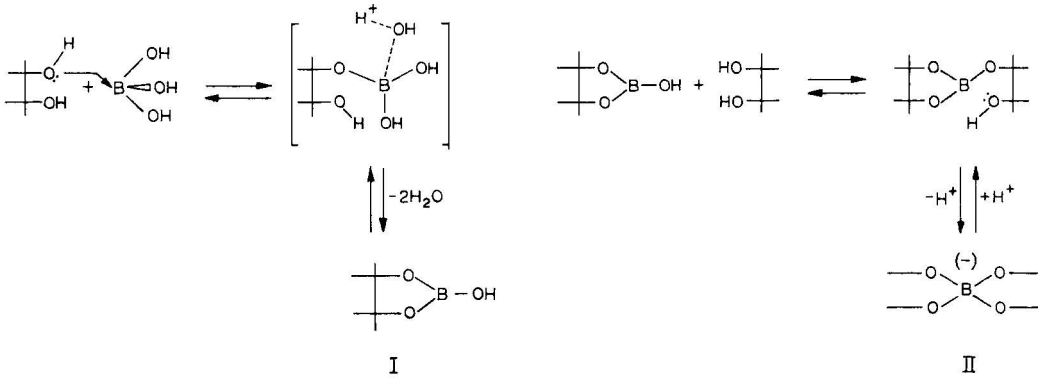


Fig. 2. Coupled-layer chromatogram of a carbohydrate mixture. Impregnation, P–R (see Table 8); solvents, ethyl acetate–2-propanol–water (2:2:1) and 32. From ref. 19.

of the neutral boric ester (I), whilst in a basic medium the complex-forming reaction proceeds until anionic complexes are formed. The conversion of the neutral ester (I) into the anionic borate (II) is dependent on the presence of a Brønsted base (proton acceptor). In order to explain the chromatographic behaviour shown in Fig. 3, we must assume that the neutral esters (I) have chromatographic characteristics similar to those of free sugars. Thus the chromatography of sugars on a thin layer of borate-impregnated silica gel must exhibit a considerable solvent dependence, since, according to the pH of the elution system, two substantially different mechanisms of separation can be obtained on the same layer, favouring a high resolution capacity.



Taking into account these points, we carried out extensive investigations of the possibilities of two-dimensional chromatographic analysis of sugars on thin layers of silica gel impregnated with borate and obtained excellent separations of carbohydrate

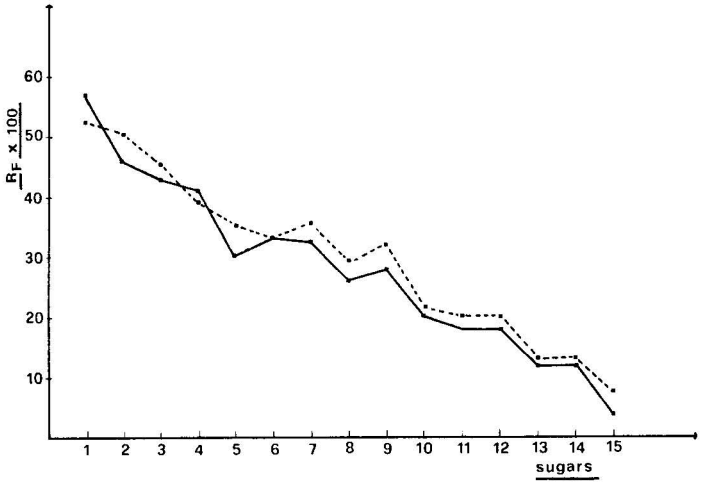


Fig. 3. $R_F \times 100$ values of carbohydrates separated on non-impregnated (— · · —) and sodium tetraborate–sodium tungstate-impregnated (———) silica gel plates. Solvent system: ethyl acetate–acetic acid–methanol–water (12:3:3:2). For description of the sugars, see Fig. 9. From ref. 18.

mixtures of clinical interest by simple and relatively brief procedures[18,27]. Mezzetti *et al.*[31] also studied molybdate and tungstate as impregnant salts in the separation of sugars. Bisulphite, which is known for its characteristic addition reactions with aldoses and chetoses, has been used for the analysis of certain monosaccharides and oligosaccharides with good results[10].

B. Cellulose

Cellulose has the same chromatographic characteristics as paper, with the advantage that elution times are shorter and the sensitivity to detection reagents is enhanced. Unlike silica gel, this support gives good resolution of carbohydrates, even without the use of impregnants, and, in addition, is superior for the analysis of oligosaccharides having high degrees of polymerization (DP).

C. Kieselguhr

Compared with silica and cellulose, the diatomaceous earth Kieselguhr has a lower water take-up and a lower surface activity. The elution systems used on this support must therefore contain less water. However, the chromatographic patterns obtained for sugars on Kieselguhr plates that have been impregnated with a specific inorganic salt are not very different from the patterns obtained on silica layers impregnated with the same salt. Furthermore, perhaps owing to its lower activity, Kieselguhr has a limited capacity, 5 μg being the maximum quantity of the sample that can be chromatographed[32]. Another of the limitations of Kieselguhr is the difficulty in revealing the sugar spots with the more common reagents. Despite the statements made by Stahl and Kaltenbach[33], anisaldehyde–sulphuric acid reagent does not show satisfactory sensitivity to the sugars on Kieselguhr.

Recently Bell and Talukder[34] reported a new reagent which is specific for the carbohydrates on Kieselguhr. We have observed that the naphthoresorcinol–sulphuric

acid reagent is sufficiently sensitive, even on Kieselguhr, when the acid and the aromatic alcohol are sprayed separately on to the plate (first a 4% solution of H_2SO_4 in alcohol, then a 0.2% solution of naphthoresorcinol in alcohol). We have also observed that 2-deoxy-sugars give dark grey spots on a Kieselguhr plate when the plate is heated to 120° for 15–20 min. However, other sugars, even if held at this temperature, do not develop any colour. These sugars are sufficiently sensitized to be readily detectable with ethanol–sulphuric acid (19:1) (ref. 27). Kieselguhr may contain organic bases or other types of impurities that may interact with certain carbohydrates at high temperatures to give chromogens.

5. ONE-DIMENSIONAL SEPARATION OF MONOSACCHARIDES, THEIR DERIVATIVES AND OLIGOSACCHARIDES

In one-dimensional TLC, the area available for the separation of sugars is approximately that between the sample application points and 70% of the height of the chromatoplate. The distribution of these substances on this area is such that only some of them can be effectively resolved.

A. Separation on cellulose

For adequate separation of carbohydrates, cellulose systems almost always require multiple elutions. In this way, the more common hexoses and pentoses can be readily separated together with certain di- and trisaccharides (Table 4). Thus the solvent system 1 of Schweiger[35] on MN 300 cellulose is useful for the analysis of the group of monosaccharides formed by galactose, glucose, mannose, fucose and rhamnose. This system needs two successive developments each of 90 min duration. Vomhof and Tucker[36] investigated solvents suitable for cellulose, but of the nine elution systems they reported only one (2) was of interest. It is worth noting that, with this solvent, D-arabinose may be distinguished from its L-isomer, the R_G values being 1.45 and 1.51. Wolfrom *et al.*[37] extended the analysis of the more common hexoses and pentoses to the methylglycosides and their amino derivatives on the microcrystalline cellulose Avirine using solvents 3 and 4. These workers also reported the separation of some α-methylglycosides from their β-enantiomers. Petre *et al.*[38] used solvent 2 on Chromedia CC-41 (W. & R. Balston, Maidstone, Great Britain) for the analysis of carbohydrates and uronic acids in plant extracts. According to these workers, 12 saccharides and three uronic acids can be separated with their system (Table 4). More recently, Raadsveld and Klomp[39] suggested solvent 5 for the complete resolution, in three successive developments, of glucose, galactose, mannose, arabinose, xylose and rhamnose, together with sucrose, maltose and lactose.

In the study of the structures of glycoproteins, glycopeptides and polysaccharides it is often important to use a chromatographic system which is capable of resolving the amino-sugars and the acetylamino-sugars, together with the most common monoses, in the hydrolyzates of the substances being studied. Although, in this respect, few results have been obtained with TLC, one can, nevertheless, mention the system butanol–pyridine–0.1 *N* hydrochloric acid (5:3:2) by which glucosamine and galactosamine, together with galactose, mannose, fucose and rhamnose, can be resolved[40]. Although the method of Günther and Schweiger[41] has given good results in the resolution of glucosamine, galactosamine and their N-acetyl derivatives, it

TABLE 4

R_G AND R_F VALUES OF SUGARS AND THEIR DERIVATIVES ON CELLULOSE CHROMATOPLATES

For solvent systems, see Table 1. R_G = R_F value relative to glucose.

Carbohydrate	*MN 300*			*Chromedia CC-41*	*Microcrystalline (Avirine) (R_F values)*	
	1	*2*	*3*	*2*	*4*	*5*
L-Arabinose		1 51				
D-Arabinose	1.11	1.45	1.31	1.30	0.46	0.31
Ribose	1.42	1.91		1.49	0.59	0.39
Xylose	1.25	1.60	1.55	1.34	0.52	0.33
Lyxose		1.70				
Galactose	0.90	0.91	0.83	0.93	0.36	0.31
Glucose	1.00	1.00	1.00	1.00	0.39	0.25
Mannose	1.09	1.23	1.15	1.17	0.44	0.30
Levulose		1.30	1.15	1.21		0.29
Sorbose		1.23				
6-Deoxygalactose	1.31			1.44		
6-Deoxymannose	1.52		2.10	1.58	0.60	0.46
α-Methyl-D-galactopyranoside					0.54	
β-Methyl-D-galactofuranoside					0.72	
β-Methyl-D-galactopyranoside					0.56	
α-Methyl-D-glucoside					0.57	
β-Methyl-D-glucoside					0.61	
β-Methyl-D-arabinoside					0.65	
α-Methyl-D-xyloside					0.70	
α-Methyl-D-lyxoside					0.75	
Sucrose		0.65	0.66	0.64		
Maltose		0.38	0.53	0.42	0.29	0.15
Cellobiose		0.32	0.30		0.25	0.13
Lactose		0.26				

loses its practical importance because these workers did not compare the separations with those of the monosaccharides which are often present together with the aminic derivatives in the analytical samples.

The one-dimensional analysis of oligosaccharides having DP 4–12 can be more effectively carried out on cellulose chromatoplates. In their study of the enzymatic hydrolysis of the complex polysaccharides of Agar, Duckworth and Yaphe[42] described the one-dimensional separation of the neoagarobioses (DP 2–12) on thin layers (Table 6). These workers used microcrystalline 0.25-mm cellulose layers (Camag, Mutenz, Switzerland) and solvent systems 7 and 8. After being separated from the neutral compounds on a column of DEAE-Sephadex A-25 (Cl^-), the ionic oligosaccharides of the Agar hydrolyzates were analyzed on cellulose using solvent system 9 in a single development.

The chromatographic system suggested by Damonte *et al.*[43] requires three successive elutions, and does not offer very good separation possibilities.

B. Separation on silica gel

The complete resolution of glucose, galactose, mannose, levulose, sorbose and

TABLE 5

$R_F \cdot 100$ VALUES OF SUGARS AND THEIR DERIVATIVES ON SILICA GEL AND KIESELGUHR LAYERS

SG(?) = Silica gel (trade mark not specified) impregnated with a 2 *M* NaH_2PO_4 solution; SG = silica gel (Merck) impregnated with 0.1 *M* sodium bisulphite; SG* = silica gel KSK impregnated with 0.3 *M* NaH_2PO_4; SG** = silica gel (Fluka DO) impregnated with 0.03 *M* boric acid; PSG = precoated silica gel (Merck, art. 5715) unimpregnated when solvent 31 is used (with solvent 32 the layer is impregnated with 0.5 *M* NaH_2PO_4, allowing the plates to stand overnight in a bath of aqueous alcohol); SG-H = non-impregnated silica gel (Merck); KGA = Kieselguhr (Merck) + 0.02 *M* sodium acetate; KGP = Kieselguhr (E. Merck) impregnated with 0.15 *M* NaH_2PO_4. The R_F values in parentheses, corresponding to the KGP/48 system, were obtained by a double development with the same solvent. For solvent systems, see Table 1.

Carbohydrate	*SG(?)*		*SG*			*SG**		*SG***				*SG-H*				*PSG*		*KGA*	*KGP*	
	10	*11*	*12*	*13*	*14*	*17*	*18*	*20*	*21*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*47*	*48*	*50*
Arabinose	28	47	32	51	63			52	55	39	33					67	47	32	25(42)	61
Ribose	37	58	50	57	69			30	35	33	25					72	60	66	39(62)	83
Xylose	40	58	34	59	68			46	48	42	39					76	63	45	38(60)	77
Lyxose			46	59	68			56	50	43	37						65			
Xylulose																	76			
Galactose	16	30	32	39	53			40	52	35	26	54	55	72	30	58	30	11	10(18)	28
Glucose	22	41	28	48	61			54	61	38	33	62	61	75	42	65	41	14	14(25)	42
Mannose	28	49	41	53	60			52	57	42	36					69	50			
Levulose	26	46	28	43	57			22	33	31	16					64	46	25	20(34)	53
Sorbose			43	47	56			16	24	25	8					69	52			
Tagatose			46	53	61												60			
Mannoheptulose								24	29	33	16									
2-Deoxyribose																86	83			
2-Deoxyglucose			68	73	79															
6-Deoxyglucose																	80			
Fucose			49	55	62			66	58	42	42					75	65	47	55(77)	91
Rhamnose	55	72	57	62	68			70	62	45	57					85	83	78	80(97)	98
α-Methylglucoside								74	67	43	53									
α-Methylmannoside								75	62	46	59									

Galacturonic acid						8	16											
Glucuronic acid						27	26											
Mannuronic acid						23	33											
Glucurone						50	55											
Mannurone						36	46											
Galactosamine												13	19	22	7			
Glucosamine												21	26	31	10			
N-Acetylglucosamine												54	59	74	33			
N-Acetylgalactosamine												45	51	66	26			
N-Acetyl-Neuraminic acid												3	5	2	1			
Sucrose	14	36	20	40	55	42	53	59	70	37	27					66	47	
Turanose								38	51	35	24							
Maltose	12	32	11	35	50	35	47	53	63	37	21					57	34	
Isomaltose																	17	
Trehalose			5	23	38	31	45	41	58	34	20					57	31	
Gentiobiose																	20	
Cellobiose						39	49									56		
Lactose	6	22	8	23	36			34	52	27	12					47	23	
Lactulose			10	27	40													
Melibiose																36	14	
Panose																35	14	
Melezitose			4	30	49											56	36	
Raffinose	4	12	4	13	28	29	40	25	47	26	8					42	17	
Maltotriose																46	26	
Isomaltotriose																25	6	

tagatose, which are the more common constituents of clinical analysis samples such as urine and plasma, and of extracts from other biological materials, is of great practical interest. The three aldohexoses can be resolved with relative simplicity (Table 5). Complications arise when the aldoses have to be examined together with the hexoketoses, since the hexoketoses generally tend to give spots which overlap those of glucose and mannose. The separation of these two groups of isomers can be achieved on boric acid-impregnated silica gel plates, although a clear-cut separation among the aldoses and ketoses is not always achieved (Table 5). With the elution systems 20, 25 and 26, the quartet galactose, mannose, fructose and sorbose can be separated on 0.03 *M* H_3BO_3-impregnated silica gel layers (Fluka, Buchs, Switzerland). Glucose can be resolved together with the other carbohydrates when solvent 21 is used[27]. Using the acetone–water (9:1) solvent system and its variants (Table 8), we were able to separate galactose, glucose, fructose, sorbose and tagatose on silica gel layers (Merck, Darmstadt, G.F.R.) (Fig. 4). Although there was overlapping of mannose and fructose, we also obtained good resolution with solvent system 23 (ref. 27).

As far the pentoses are concerned, it has been observed that arabinose and ribose can usually be resolved easily also in the presence of the aldohexoses (Table 5). Arabinose, ribose and xylose are clearly resolved, together with glucose and galactose, by the chromatographic system of Ragazzi and Veronese[14], *i.e.*, silica, 2 *M* NaH_2PO_4 and solvent 10 or 11. Adachi[10] reported good separations of these three compounds, as well as of other monosaccharides and oligosaccharides, by use of silica gel impregnated with 0.1 *M* sodium bisulphite and solvent 12. Lombard[44] described a method which necessitates multiple successive elutions for the complete separation of the four aldopentoses, together with fructose, rhamnose and glucose, in 1 *N* H_2SO_4 solution. This worker used silica gel G (Merck) impregnated with a Soerensen phosphate buffer (pH 8), and methyl ethyl ketone–acetic acid–saturated solution of boric acid (9:1:1) as the solvent system. Complete separation of arabinose, ribose, xylulose and ribulose can be obtained on silica gel impregnated with 0.35 *M* NaH_2PO_4 by use of solvents 27, 28 and 29. By use of the same systems, the deoxysugars fucose, rhamnose, 2-deoxyglucose and 2-deoxyribose can also be resolved on phosphate-impregnated silica gel (Fig. 4).

The analysis of the more common uronic acids can be carried out with solvent systems 17 and 18 on silica gel KSK impregnated with monobasic sodium phosphate (0.3 *M*) (ref. 17).

Until now, there have been few results on the chromatographic analysis of amino-sugars on thin layers of silica gel, owing to the practical necessity of a clear-cut resolution of the more common amino derivatives, together with the hexoses and non-amino derivatives, including glucose, mannose, galactose, fucose, rhamnose, glucuronic acid and mannuronic acid, which are the most frequently occurring constituents in the hydrolyzates of glycoproteins and mucoid substances. The elution systems that, generally, give good separations of the last substances, do not cause the amino-sugars to migrate satisfactorily on silica gel impregnated with phosphates or borates. Some workers have used silica gel supported on glass fibre and impregnated with monobasic phosphate[45] or copper sulphate[46] for the separation of this class of compounds. However, their ammonium-containing solvent systems were unsuitable for the simultaneous study of the neutral monosaccharides and the aminic derivatives, because the silica gel catalyzes the amination reaction of the monosaccharides in the

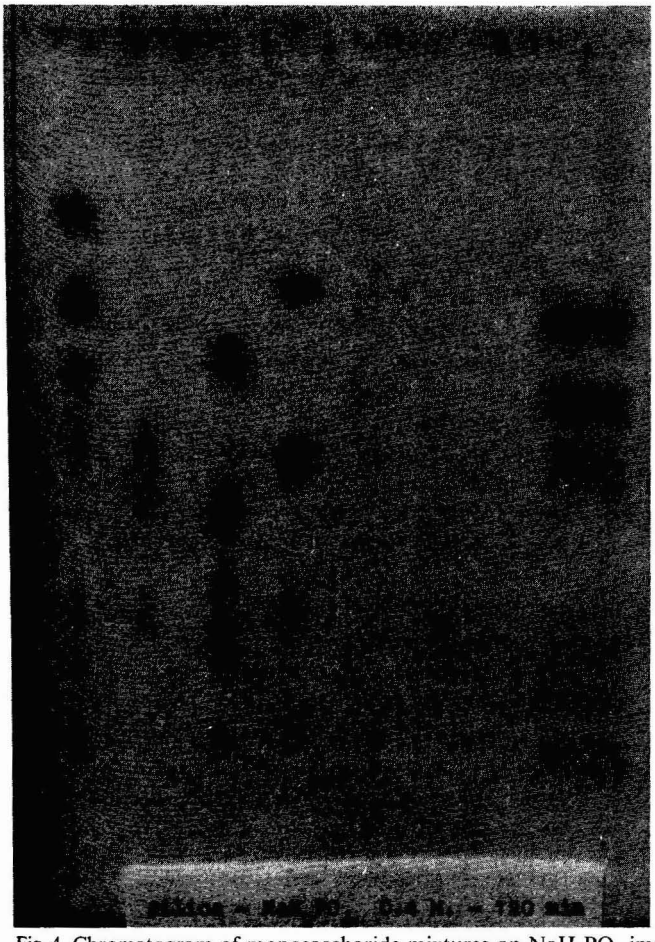


Fig. 4. Chromatogram of monosaccharide mixtures on NaH_2PO_4-impregnated silica gel 60 (Merck). Solvent system, 28; development time, 2 h; spray reagent, NPR. Samples (from left to right and from the lower part of the chromatogram): galactose, glucose, mannose, fucose, 2-deoxygalactose, 2-deoxyglucose, rhamnose, digitoxose; arabinose, ribose, fucose; galactose, glucose, fructose, sorbose, tagatose, 2-deoxygalactose; galactose, mannose, ribulose, xylulose; galactose; mannose. From unpublished results of Lato *et al.*[27].

presence of the ammonium ions. Thus the neutral monosaccharides migrate on the chromatoplate in part unchanged and in part as their amino derivatives, thereby giving double spots[46–48].

Non-impregnated silica gel layers, therefore, seem to be more suitable for the separation of amino-sugars, even if the number of monoses that can be examined by this method is somewhat limited. The study of Gal[49] on the separation and identification of the amino-sugars galactosamine and glucosamine and their N-acetyl derivatives, together with glucose, galactose and the N-acetyl neuraminic acid, on a non-impregnated silica gel layer is noteworthy in this context.

As far as the chromatographic examination of polyalcohols is concerned, the one-dimensional method of Weinstein and Segal[50] gave good resolution of sorbitol,

galactitol and mannitol, in addition to galactose, glucose and mannose. The analysis was performed on a silica gel layer impregnated with a borate buffer (0.02 *M* $Na_2B_4O_7$ + 0.02 *M* H_3BO_3); the elution solvent was 2-propanol–water (4:1) in a triple development. The method used by Němec *et al.*[51], which enabled separation of a large number of aldonic acids and polyhydroxy alcohols on a non-impregnated silica gel, seems to be more rapid and more efficient.

The use of impregnants in TLC represents a considerable limitation in the analysis of oligosaccharides having DP < 3. However, for the lighter saccharides, impregnated sorbents give high selectivity and permit the resolution of numerous di- and tri-saccharides. Thus, the system silica–0.1 *M* $NaHSO_3$–solvent 13 is suitable for the analysis of saccharides, including lactose, lactulose, maltose, saccharose and raffinose. Solvent 14 gives an equally good separation of these oligosaccharides when used on bisulphite-impregnated silica gel, although the resolution of the monoses is somewhat poorer[10]. Jeffrey *et al.*[52] described a technique that, besides offering the possibility of analysis of certain monosaccharides, gave a rapid one-dimensional resolution of cellobiose, saccharose, maltose, gentiobiose, lactose, melibiose and raffinose. The chromatographic system was silica gel–0.02 *M* boric acid–solvent 19 or 20. The non-aqueous solvent 19 caused spot diffusion, which these workers tried to avoid by a double development of the plate in the same solvent.

Mezzetti *et al.*[31] conducted a systematic study of solvents, sorbents and impregnants suitable for the separation of common di- and tri-saccharides. The best systems permitted the resolution of 9–12 oligosaccharides. However, the long time (8–20 h) needed for the entire chromatographic process seriously limited its adaptability to rapid routine analysis. The oligosaccharides isomaltose, maltose, panose, maltotriose, isomaltotriose and saccharose, as well as some monosaccharides, can be examined by the Hansen method[53] (Fig. 5). The separation is carried out on ready-made silica gel plates (Merck, art. No. 5715) impregnated with monobasic sodium phosphate; the plates are immersed for 15–20 h in a bath of a 0.5 *M* NaH_2PO_4 solution. The elution system is 2-propanol–acetone–0.1 *M* lactic acid (2:2:1).

The separation and identification of heavy fragments produced by the hydrolysis of polysaccharides cannot be carried out with the systems so far described, owing to the low migration rate of these saccharides on silica layers impregnated with inorganic salts. Non-impregnated silica gel layers and solvents with a relatively high water content are the most suitable systems for the chromatographic examinations of these compounds[54–56]. Powning and Irzykiewicz[54] used silica gel G plates (Merck) and solvents containing 25–30% water (Table 6) in their chromatographic studies on the hydrolyzates of chitin. These workers found a linear relation between the R_M values of the saccharides examined and their DP values, thus showing that the separation process for these compounds is based mainly on partition. On the other hand, with the system used[56] for the identification of the oligosaccharides produced by starch hydrolysis, there does not seem to be a similar relation; the isomers maltotriose, isomaltotriose and 6-α-glucosylmaltotriose, as well as maltotetraose and isomaltotetraose, gave different R_F values (Table 6).

C. Separation on Kieselguhr

The limited number of reports on the chromatography of sugars on thin layers of diatomaceous earth can, perhaps, be attributed to the poor capacity of this sorbent.

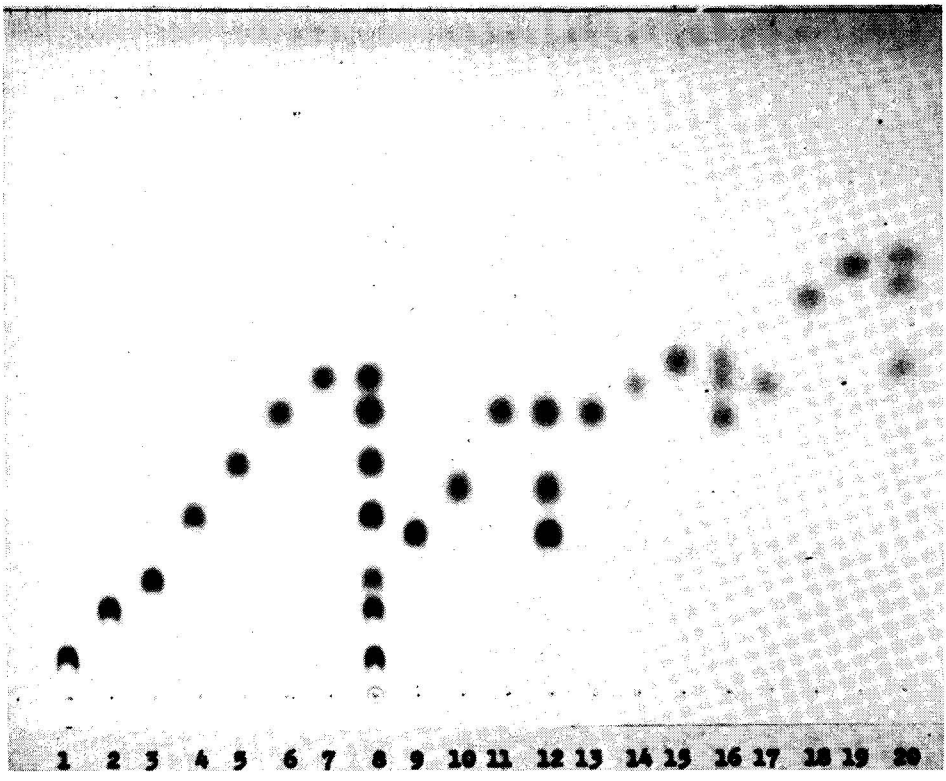


Fig. 5. Chromatogram of mono-, di- and trisaccharides on 20 × 20 cm pre-coated TLC silica gel 60 plates (Merck, Art. No. 5715) impregnated with NaH_2PO_4. Solvent system, acetone–isopropanol–0.1 *M* lactic acid (40:40:20); spraying reagent, aniline–diphenylamine–acetone–80% H_3PO_4 (4 ml:4 g:200 ml:30 ml). Samples, 1 μl of (1) isomaltotriose, (2) panose, (3) isomaltose, (4) maltotriose, (5) maltose (6) glucose, (7) sucrose, (8) 1–7, (9) lactose, (10) galactose, (11) glucose, (12) 9–11, (13) glucose, (14) fructose, (15) mannose, (16) 13–15, (17) arabinose, (18) ribose, (19) xylose, (20) 17–19. From ref. 53.

However, Kieselguhr offers a separation of monoses which is comparable to that obtained on silica gel (Table 7).

Kieselguhr G (Merck) impregnated with 0.15 *M* monobasic sodium phosphate permits the resolution of the monoses galactose, glucose, fructose, arabinose, xylose, fucose and rhamnose with solvents 34, 35 and 37. Double development increases the degree of separation without altering the quality of the spots. Xylose and ribose can be resolved by use of solvent system 37 in a double development on a 20 × 35-cm plate[57]. Furthermore, the elution system 38 applied on Kieselguhr chromatoplates (Merck) has given excellent separations of hexoses, pentoses and deoxy-sugars in our laboratory. These results could not, however, be reproduced with impure samples, for example urine.

Shannon and Creech[58] showed that Kieselguhr is very suitable for the chromatographic separation of oligosaccharides having high DP values. The fact that these workers stated that they were unable to reproduce the results previously obtained by Weil and Hanke[59] and Huber *et al.*[60] on the same support suggests that although Kieselguhr gives interesting results it is not suitable for standard methods.

TABLE 6

R_G AND $R_F \cdot 100$ VALUES OF SOME OLIGOSACCHARIDES (DP 2–16) SEPARATED ON CELLULOSE, SILICA GEL AND KIESELGUHR

NAG- = N-Acetylglucosamine. For supports see Table 5. For solvent systems, see Table 1. $R_{Gal} = R_F$ value relative to galactose.

Saccharide	*Microcrystalline cellulose*			*PSG*	*SG-H*								*KG*	
	7	*8*	*9*	*31*	*37*	*38*	*39*	*40*	*41*	*42*	*43*	*44*	*45*	*46*	*52*	*53*
Cellobiose				56	33	36										
Neoagarobiose	1.11		1.10													
NAG-biose							63	45	58	39	57	41	42	27		
Maltobiose				57											92	89
Cellotriose					23	30										
NAG-triose							42	24	48	29	48	32	30	16		
Maltotriose				46											86	84
Cellotetraose					15	23										
Neoagarotetraose	0.87		0.80													
NAG-tetraose							33	17	39	21	40	23	22	10		
Maltotetraose				34											80	81
Isomaltotetraose				17												
Cellopentaose					8	18										
NAG-pentaose							25	12	31	15	32	17	14	6		
Maltopentaose				27											74	77
Cellohexaose					5	13										
Neoagarohexaose	0.65		0.51				19	8	23	11	25	11	9	3		
NAG-hexaose																
Maltohexaose				23											69	73
Maltoheptaose															64	68
Maltooctaose															58	62
Neoagarooctaose	0.41	0.57	0.32													
Maltononaose															50	55
Maltodecaose															44	49
Neoagarodecaose		0.42	0.18													
Malto – 11 DP															35	42
Malto – 12 DP															29	36
Neoagaro – 12 DP		0.30														
Malto – 13 DP															22	29
Malto – 14 DP		0.18													18	24
Neoagaro – 14 DP																
Malto – 15 DP															15	20
Malto – 16 DP															12	16

TABLE 7

$R_F \cdot 100$ VALUES OF SUGARS AND THEIR DERIVATIVES ON SILICA GEL AND KIESELGUHR LAYERS[27]

SG.B = Silica gel (Merck) impregnated with 0.1 *M* boric acid; SG.P = silica gel (Merck) impregnated with 0.2 *M* NaH_2PO_4; SG.P* = silica gel (Merck) + 0.3 *M* NaH_2PO_4; SG.P** = silica gel (Merck) + 0.35 *M* NaH_2PO_4; KG.P = Kieselguhr (Merck) impregnated with 0.3 *M* NaH_2PO_4. For solvent systems, see Table 1.

Carbohydrate	*SG.B*	*SG.P*			*SG.P**		*SG.P***	*KG.P*	
	26	*23*	*26*	*28*	*28*	*29*	*27*	*48*	*51*
Galactose	20	15	8	22	12	11	12	10	6
Glucose	24	21	12	33	20	21	21	18	12
Mannose	26	27	18	43	28	27	30	26	23
Levulose	12	25	17	41	26	26	27	28	23
Sorbose	6	30	18	47	32	33	32	31	29
Tagatose	12	25	34	55	43	43	40	38	41
Mannoheptulose		21	13	36	27	25	24		
Sedoheptulose		22	13	40	30	31	25		
Arabinose	25	27	17	42	30	30	28	29	26
Ribose	18	38	32	59	47	46	43	46	44
Xylose	26	39	32	61	49		45	44	46
Lyxose	28	39	35		52	50	48	46	50
Ribulose	3	38	38	62	54	58	48		53
Xylulose	2	45	48	70	65	66	60		61
2-Deoxyribose	46	55	58	75	73	72	66	88	87
2-Deoxygalactose	36	40	41	65	60	58	55	63	68
2-Deoxyglucose	45	50	52	72	69	67	62	78	80
Rhamnose	40	55	58	75	73	72	66	87	87
Fucose	28	34	30	58	51	50	47	60	63
Digitoxose	58	73	75	83	81	82	77		

6. TWO-DIMENSIONAL SEPARATION

Two-dimensional chromatography usually implies amphoteric behaviour of the substances under examination, in the sense that such substances should exhibit distinct characteristics in the two successive developments, and produce a wide distribution of spots over the chromatographic plate. Such a requirement is, obviously, related to a difference between the solute–solvent and/or solute–support interactions in the two successive chromatographic processes, and this can be easily obtained for substances which are naturally amphoteric, such as the amino acids and the indole derivatives that migrate on the chromatoplate both as positively charged molecules and negative ions, according to the pH of the elution system. In this case the separation depends on the p*K* values.

For non-amphoteric substances, such as carbohydrates, a similar differentiation in the two developments is feasible on the borate-impregnated silica gel layers previously discussed. Juxtaposition of two layers of differently impregnated silica gel produces a good separation of monosaccharides and their derivatives and oligosaccharides, because the migration of the sugars takes place in each of the two layers according to different chromatographic mechanisms. The results obtained with this technique are summarized in Table 8. Despite the fact that these separations are inter-

TABLE 8

CHROMATOGRAPHIC DATA FOR SUGARS SEPARATED ON COUPLED LAYERS OF SILICA GEL G (FLUKA DO), IN A TWO-DIMENSIONAL TECHNIQUE

Impregnants: P = 0.132 *M* sodium tetraborate–0.204 *M* boric acid–0.06 *M* sodium tungstate (1:1:1); Q = 0.036 *M* boric acid; R = 0.24 *M* sodium acetate; S = 0.24 *M* monobasic sodium phosphate; T = saturated solution of molybdic acid. Solvents: A = *n*-butanol–ethyl acetate–2-propanol–acetic acid–water (7:20:12:7:6); B = ethyl acetate–2-propanol–water (10:6:3); C = 2-propanol–*n*-propanol–water (14:14:5); D = methyl acetate–2-propanol–water (2:2:1). The remaining solvents are defined in Table 1.

Impregnant		*Solvent system*		*No. of sugars separated*	*Comments*
Narrow layer	*Wide layer*	*First run*	*Second run*		
P	Q	D	A	26	Generally, chromatography on P–Q coupled layers results in glucose–mannose and maltose–trehalose–cellobiose overlapping; nevertheless fair separation of many hexoses, pentoses and disaccharides is achieved. Solvent 12 and A are almost equivalent with respect to their resolution capacity, but the former elutes more rapidly (1.5 h) than the latter (3–4 h)
		D	12	34	
		24	A	28	
		24	12	31	
		24	12	31	
		C	A	28	
		C	12	28	
P	R	24	A	32	Maltose–trehalose overlap, and so do arabinose–glucose and galactose–sedoheptulose
		24	12	31	
		C	A	24	
		C	12	26	
		D	A	32	
		D	12	25	
R	P	22	D	28	The solvents of the first run have the advantage of moving at very fast rates (60–90 min); some oligosaccharides give double spots
		20	D	34	
T	Q	13	12	22	Sharply defined spots
		B	A	20	
T	R	B	12	20	Slightly diffuse spots
		B	22	22	
		13	20	22	
T	S	13	20	19	Slightly diffuse spots
		B	20	21	
		B	22	22	
R	T	22	B	21	Solvent systems 22–13 and 20–13 give sharply defined spots
		22	13	22	
		20	B	23	
		20	13	21	

esting (Fig. 2), the length of time required for the double development (7–8 h) is a limiting factor.

A new method reported by Lato and his co-workers[18,27] overcomes this limitation while retaining the same type of separation (Fig. 6). It is based on the different properties of the borate-impregnated silica gel layer according to the pH of the eluent systems (Fig. 7). The results obtained by Günther and Schweiger[41] in the two-dimensional separation of certain amino-sugars are, in our opinion, based on the same principle, since between the first and the second chromatographic runs the cellulose chromatoplate is sprayed with a borate buffer at pH 8.

7. VISUALIZATION OF THE SUGAR SPOTS

There is a large number of reagents for the detection of carbohydrates on thin layers and these have been well documented in various papers and reviews[10,14,31,43,44,61,62]. Here we report only those reagents which, because of their sensitivity, specificity and capacity for polychromatic staining, are useful not only for revealing the sugars but also for their identification.

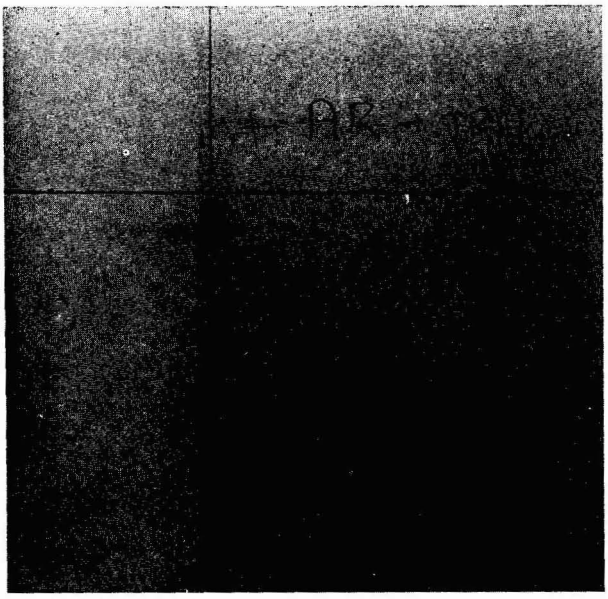


Fig. 6. Two-dimensional chromatogram of mono- and oligosaccharides on tetraborate–phosphate (0.01 *M* H_3PO_4 + 0.028 *M* $Na_2B_4O_7$)-impregnated mono-layer of silica gel 60 (Merck). Solvent systems, 24–30. From unpublished results of Lato *et al.*[27].

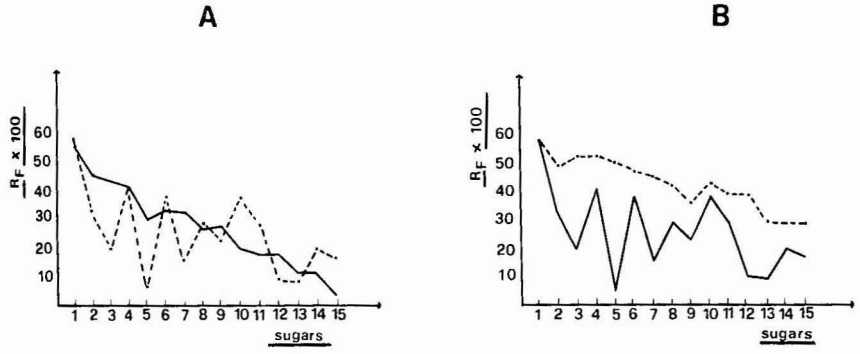


Fig. 7. A, $R_F \times 100$ values of sugars separated on a sodium tetraborate–sodium tungstate-impregnated silica gel layer by the neutral solvent 24 (– – –) and by the acidic solvent 12 (———). B, $R_F \times 100$ values of the same sugars separated on a sodium tetraborate–sodium tungstate-impregnated silica gel layer (———) and on a non-impregnated silica gel layer (– – –) by solvent 24. For description of the sugars, see Fig. 9. From ref. 18.

TABLE 9

DETECTION OF SUGARS AND THEIR DERIVATIVES ON CHROMATOGRAPHIC THIN LAYERS

For detection reagents, see Table 10; col. = colours developed; sens. = sensitivity limits in μg. Bl = blue; V = violet; PuR = purple red; Lg = light; Gr = green; Pk = pink; Or = orange; Y = yellow; Br = brown; Ol = olive; I = intense; Bk = brick; Gy = grey; F = faint. (———), not reported in the original reference. The reaction colours in ref. 27 were obtained on silica gel (Merck); solvent systems which did not contain acetic acid or pyridine were used as elution systems.

Sugar	*NPR*[27]		*DPA*[43]	*DPA*[27]	*APT*[43]	*APT–NPR*[27]		*TBA*[27]	
	col.	*sens.*	*col.*	*col.*	*col.*	*col.*	*sens.*	*col.*	*sens.*
Galactose	Bl	1.5	Gy	BlGy	Y	YBr	0.5	LgBl	2.0
Glucose	V	4 0	GyBl	BlGy	Y	YBr	0 5		
Mannose	Bl	1.5	BlGy	BlGy	Y	YBr	0.5		
Fructose	PuR	0.5	YOr	LgR		PuR	0.1	Y	0.5
Sorbose	PuR	0.1	—	Y	—	PuR	0.1	Y	0.5
Tagatose	R	0.5	—	LgR	—	PuR	0.1	Y	0.5
Mannoheptulose	PuR	0.5	—	PuR	—			Y	0.1
Sedoheptulose	PuR	4.0	—	PuR	—			Y	2.0
Arabinose	LgBl	1.5	Ol	FGyBl	Pk-BkR	BrY	0.5		
Ribose	LgBl	1.5	Ol	FGyBl	Pk-BkR	BrY	0.5		
Xylose	Br	1.5	Ol	IGyBl	Pk-BkR	BrY	0.5		
Lyxose	LgBl	1.5	Ol	IGyBl	Pk-BkR	BrY	0.5		
Ribulose	Gr	0.5	—	Y	—	Or	0.1	Y	4.0
Xylulose	Or	0.2	—	Y	—	Or	3.0	Y	4.0
2-Deoxyribose	PeaGr	0.5	Pk	Br-IV*	Y	BlGr	1.0		
2-Deoxygalactose	LgBl	0.5	—	V-IBr*	—	Gr	2.0		
2-Deoxyglucose	LgBl	0.5	—	V-IBr*	—	Gr	2.0		
6-Deoxymannose	Pk	0.5	YGr	Ol-IGy*	Y	BrY	1.0		
6-Deoxygalactose	Pk	0.5	—	Ol-IBl*	—	BrY	1.0		
Galacturonic acid			—		—				
Glucurone			—		—				
α-Methylglucoside	Bl	0.5	—		—	R	2.0		
α-Methylmannoside	Pk	0.5	—		—	Bl	1.5		
α-Methylarabinoside	Bl	0.5	—		—	V	0.5		
α-Methylxyloside	Bl	0.5	—		—	Bl	0.5		
Sucrose	PuR	0.5	BrY	Or-V*		R	0.5	Y	
Maltose	Bl	4.0	Bl	Bl		BrY	1.0		
Lactose	Bl	4.0	GyBl	Bl	Y	BrY	1.0		
Melibiose	Bl	4.0	GyBl	Bl	Y	BrY	1.0		
Turanose	PuR	0.2	YBr	R		R	0.5		
Raffinose	PuR	0.5	YGr	V		R	0.5		

* Colours immediately after detection and some hours after detection.

The furfural-yielding reagents, such as naphthoresorcinol–sulphuric acid[20,63], α-naphthol–phosphoric acid[62], orcinol–hydrochloric acid[62], aniline–diphenylamine–phosphoric acid[62], *p*-anisidine–phthalate[35,44], aniline–phthalate and aniline–phosphate[64], under specific conditions, permit the recognition, through colour differences, of spots belonging to various classes of carbohydrates. Furthermore, under certain conditions, anisidine–phthalate and aniline–phthalate reagents are specific for aldoses, since ketoses and oligosaccharides containing ketoses do not show marked reactivity at low concentrations (10 μg). The limit of sensitivity of these reagents to aldoses is 0.5 μg.

APT[27]		*TBA–NPR*[27]		*URE*[27]		*URE–NPR*[27]		*AMP*[27]		*ANP*[27]	
col.	*sens.*	*col.*	*sens.*	*col.*	*sens.*	*col.*	*sens.*	*col.*	*sens.*	*col.*	*sens*
Y	0.5	LgBl	2.0			Bl	0.5				
Y	0.5	LgBl	2.0			Bl	0.5				
Y	0.5	LgBl	2.0			Bl	0.5				
Y	4.0	BrY	0.5	Bl	0.5	PuR	0.5	Y	1.0		
Y	4.0	BrY	0.5	Bl	0.5	PuR	0.5	Y	1.0		
Y	4.0	BrY	0.5	Bl	0.5	PuR	0.5	Y	1.0		
				BrY	1.0			Gr	1.5	V	1.0
								Gr	1.5	V	1.0
BkR	0.5	LgBl	1.0							Pu	0.5
BkR	0.5	LgBl	1.0							Pu	0.5
BkR	0.5	LgBl	1.0							Pu	0.5
BkR	0.5	LgBl	1.0							Pu	0.5
Y	3.0	V	0.1	BrY	0.5			Y	0.5	Bl	0.5
Y	3.0	V	0.5	BrY	0.5			Y	2.0	Bl	2.0
Y	3.0	BrY	2.0					Bl	3.0	Or	2.0
Y	3.0	BrGr	2.0					Bl	0.5	Or	2.0
Y	3.0	BlGr	2.0					Bl	0.5	Or	2.0
Y	1.0	V	2.0							Or	0.5
Y	1.0	V	2.0							Or	0.5
				Bl	0.5			BlV	1.0		
				Bl	0.5			BlV	1.0		
				Bl	0.5						
Y	2.0										
Y	2.0										
Y	2.0										
				Bl	1.0						
				Bl	1.0						

Dimedone[65], thiobarbituric acid[66] and urea[67] form another category of detection agents which are of considerable interest owing to their selectivity for ketoses, which are easily detected even at levels of 0.5 μg, whilst the lower limits of sensitivity for the aldoses is considerably higher.

The data necessary for the identification of the spots after their visualization on the chromatoplate are shown in Table 9. It should be borne in mind that if the temperature and duration of exposure of the plate to the heat source are not exactly reproduced, both the colour, essential for the identification of the sugars, and the selectivity of the reagents may be absent. For example, when the chromatoplate is

sprayed with the naphthoresorcinol–sulphuric acid reagent and heated for more than 15 min the carbohydrates, invariably, give red spots. Colour differentiation is influenced by the amount of acid, as well as by the type of acid. The concentration of phosphoric acid in the thiobarbituric acid reagent[66] is critical for the selectivity of ketoses and deoxy-sugars. High levels of phosphoric acid also cause the aldoses to give coloured spots. The naphthoresorcinol reagent becomes inefficient for the differentiation of ketoses and aldoses when the acid content is greater than 4–5%. When the same reagent is acidified with trichloroacetic acid instead of sulphuric acid it becomes specific for the visualization of uronic acids and ketoses[32].

Another factor which may influence the specific coloration is the eluent composition. Trace amounts of some components of the elution systems, such as acetic acid, pyridine and butanol, have a tendency to persist on the layer, even after the plate has undergone heating. These trace solvents may cause the limit of sensitivity of the sugars to be raised, or may alter the colours produced after the reaction. For this reason, the anisidine–phthalate reagent, which according to Schweiger[35] should give a green coloration with hexoses, caused the aldohexoses to give similar yellow spots to the deoxy-sugars in the experiments of Damonte *et al.*[43]. We tested this reagent on a silica G layer (Merck) impregnated with monobasic phosphate, after elution with solvent system 17, and obtained the same colorations as reported by Damonte *et al.* However, the sensitivity to ketoses and ketose-oligosaccharides was equal to or somewhat higher (2.5 μg) than that of the aldoses.

Other reagents, according to our experience, are less sensitive and do not detect the special category of carbohydrates for which they were proposed. According to Stahl and Kaltenbach[32], the anisaldehyde reagent should reveal sugars at a concentration of 0.5 μg on silica or Kieselguhr layers; however, under the conditions of Bell and Talukder[34], the same chromatographic system did not give good results. We tested this reagent and obtained poor results[27]. According to Brockman *et al.*[68], the *o*-aminophenol reagent should visualize the amino-sugars. Under our conditions, this reagent was useful for the differentiation of the deoxy-sugars (Table 9), but its sensitivity to hexoses, oligosaccharides and amino-sugars was too low. Amino-sugars are, on the other hand, not only sensitive to ninhydrin and the Morgan–Elson reagent, but also to anisidine–phthalate.

8. IDENTIFICATION OF THE SPOTS

The R_F values given by the different chromatographic methods assume importance in the identification of the spots only when they are evaluated together with other criteria such as the selectivity of the detection reagent, the differences in colorations and the use of internal standards. The R_F values are not constant parameters, but vary from one experiment to another with the variations in temperature and humidity of each laboratory, according to the mark or the batch number of the silica gel used and with many other factors such as irregularities in the thin layer, tiny differences in the thickness of the layer, etc. Thus R_F values have only an indicative value, and each group of workers must adapt the chromatographic methods described to his own conditions in order to obtain good results.

In the identification of sugars by means of chemical reactions on the thin layers, one of the problems that can be most easily solved is the differentiation of the

TABLE 10

DETECTION REAGENTS FOR SUGARS AND THEIR DERIVATIVES ON THIN LAYERS OF SILICA AND CELLULOSE

Reagents		*Treatment*	*Reference*
NPR	(a) 0.2% Naphthoresorcinol in ethanol (b) Conc. H_2SO_4 Mix (a) and (b) before use (1:0.04)	100°, 5 min For revealing sugars on Kieselguhr (E. Merck) first spray with (a) and heat the plate to 120° for 10–15 min, then spray with (b) and heat to 100° for 5 min	20, 63 27
DPA	(a) 2% Diphenylamine in acetone (b) 2% Aniline in acetone (c) 85% H_3PO_4 Mix (a), (b) and (c) before use (5:5::1)	100°, 10 min	43, 62
APT	0.1 *M* solution of *p*-anisidine and phthalic acid in 96% ethanol	100°, 10 min	35
APT–NPR		First spray with APT, heat to 100° for 10 min, record the developed colours and then spray with NPR and heat to 100° for 5 min	27
TBA	(a) 0.5% Thiobarbituric acid in ethanol (b) 85% H_3PO_4 Mix (a) and (b) (1:0.02)	100°, 10 min	66
TBA–NPR		First spray with TBA, revealing the ketoses, then spray with NPR	27
URE	5 g of urea in 20 ml of 2 *M* H_2SO_4 Mix with 100 ml of ethanol. (The original urea reagent contained HCl instead of H_2SO_4, refs. 32 and 67)	100°, 20–30 min	27
URE–NPR		First spray with URE, heat and then spray with NPR	27
AMP	1% *o*-Aminophenol in methanol + 10 ml of 85% H_3PO_4 + 5 ml of water. This reagent was originally devised for hexosamines[68]. Under our conditions, it was useful for detecting pentoses and deoxy-sugars	100°, 10 min	27
ANP	10 vol. 0.5% α-Naphthol in 50% ethanol + 1 vol. H_3PO_4	90°, 10–15 min	62

ketose series from the aldose series. This is made possible by the availability of reagents which are specific for only one of these two series of carbohydrates, and of reagents which reveal the sugars of both series by means of two distinctly different colours (Tables 9 and 10). Aminoguanidine sulphate has been proposed[69] as a new reagent for the selective differentiation of fucose. We have tested[27] urea–sulphuric acid (Table 10), a variant of the reagent specific for ketoses and uronic acids[32], and have found it to be equally useful for the differentiation of 2-deoxy-glucose and 2-deoxy-galactose from fucose, rhamnose and 2-deoxy-ribose, as well as from pentoses and hexoses. We have also found[27] that the *o*-aminophenol reagent[68] is suitable for the selective detection of 2-deoxy-glucose and 2-deoxy-galactose; rhamnose and fucose do not react. The series of 2-deoxy-sugars is also easily detected by use of α-naphthol–sulphuric acid which gives colours which are clearly distinct from those of

the other sugars; naphthoresorcinol–sulphuric acid is useful for the detection of ketoses from both their aldo-isomers and other sugars.

An interesting procedure of successive coloration is the association of reagents specific for the aldoses with those for ketoses, and the multiple-coloration technique of Damonte *et al.*[43] offers very good possibilities for the identification of carbohydrates.

The identification of the spots in two-dimensional chromatoplates is facilitated by the use of internal standards made up of substances which are, if possible, not saccharides. Such standards, as they occupy known positions on the chromatoplate, can be used as fixed points of reference. Straight lines traced through such points will divide the chromatogram into restricted areas and the groups of sugars can be easily characterized. In a recent paper[18] we suggested a method for identifying urinary sugars on two-dimensional chromatograms by use of urea and sucrose, occurring naturally in the analysis samples, as internal standards (Fig. 8). Urea can be used as internal standard because: (a) although it is not a saccharide, it reacts with the naphthoresorcinol–sulphuric acid reagent and yields a very distinct colour; (b) it does not cause negative interference with the chromatographic migration of the sugars; (c) it does not cause overlapping with the sugars examined and so mask their

Fig. 8. Two-dimensional chromatogram of untreated urine from a subject affected by an intestinal absorption disorder. Sorbent: sodium tetraborate–sodium tungstate (0.024 *M* $Na_2B_4O_7$ + 0.0125 *M* Na_2WO_4)-impregnated mono-layer of silica gel G 60 (Merck)–syloid 63 (W. R. Grace) (2:1, w/w). Solvent systems 24–12. From unpublished results of Lato *et al.*[27].

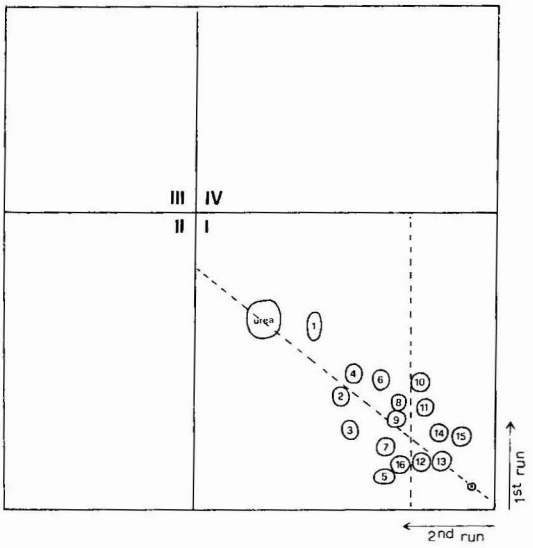


Fig. 9. Two-dimensional chromatogram of urea and sugars of clinical interest on sodium tetraborate–sodiumtungstate-impregnated silica gel mono-layer. Solvent systems 24–12. Spots: 1 = 2-deoxyribose; 2 = xylose; 3 = ribose; 4 = fucose; 5 = xylulose; 6 = glucose; 7 = fructose; 8 = galactose; 9 = sedoheptulose; 10 = sucrose; 11 = maltose; 12 = palatinose; 13 = lactulose; 14 = lactose; 15 = raffinose; 16 = allulose. From ref. 18

visualization. Sucrose has, equally, a chromatographic migration that makes it possible to trace two ideal straight lines at a tangent to the sugar spot, thereby outlining a zone (Fig. 9) in which only oligosaccharides are found.

9. QUANTITATIVE DETERMINATION

Two different procedures can be used with reasonable accuracy to determine well separated sugars on thin layers: (a) analyses *in situ*, in which the quantitative evaluation is carried out directly on the chromatoplate after the spots have been visualized with the appropriate reagent; (b) spectrophotometric analyses after elution of the spots from the solid chromatographic medium.

Of the quantitation methods *in situ*, those based on visual evaluation of the spots for comparison with external standards are unsatisfactory because they result in subjective and approximate responses. Densitometric methods are sufficiently exact and sensitive, although they present some technical difficulties, owing to the irregularities of the spots and the poor reproducibility of the spray detection technique which invariably leads to small differences in the intensities of the spots not associated with the amount of the sugar being tested. For these reasons, the application of these methods is limited to the determination of the spots in one-dimensional chromatograms[70–73].

The spectrophotometric determination of the eluates is more reliable, since the operations required for this technique, even if relatively long and laborious, involve less parameters and error. Furthermore, the quantitative determination of mannose and fructose, the chromatographic separation of which is difficult (Tables 4 and 5),

can be easily performed, even when these compounds are both present on the chromatoplate[39]. The total amount of both of these sugars can be determined with the help of a general reagent (*e.g.*, tetrazole blue) and then fructose alone can be quantitated by the resorcin method[74]. The applications of this method to quantitative analyses are more numerous and they can be used for a large number of carbohydrates. Scott[62] has made a comprehensive illustrated summary of these techniques.

For removing samples from the chromatoplates, the area containing the substances under examination is localized by use of pure standards which are co-chromatographed on to guide strips on the same layers. The detection of these standards is then carried out by heating only the guide strip with a suitable electric resistance slide after the reaction solution has been deposited on the strip by use of a 0.1-ml syringe[75]. Another method of revealing the external standards, without contaminating the substances to be examined, is that of masking the portion of the chromatoplate containing the sample with a glass plate and warming it with a hair-dryer until the spots become visible[76]. After having sprayed only the guide strips, McKelvy and Scocca[77] heated the plate to 100° for a few seconds; the short time of exposure to the heat presumably prevented thermodegradation of the samples. However, the use of a silver salt, *e.g.*, Ag_2CO_3, avoids heating of the plate and, therefore, possible degradation of the carbohydrates.

The use of pure external standards for locating the substances under examination does not always guarantee an acceptable degree of precision, especially when one is dealing with impure biological samples. Inorganic salts such as chlorides may cause interference with the chromatographic process by altering the R_F values of the sample sugars with respect to the pure standards. It should be noted that the use of these procedures is limited to one-dimensional chromatography. On the other hand, Jeffrey *et al.*[52] and Bell and Talukdar[34] visualized the sugars under examination directly on the chromatoplate, and eluted the coloured products for spectrophotometric determination; this has the advantage of greater precision in locating the spots. The reaction product must, however, be stable in order to obtain satisfactory results.

In both methods, the possibility of a quantitative elution of the sample from the chromatographic gel is of major importance, in the sense that a *ca.* 100% recovery aids the sensibility of the methods. Scott's studies[75] on the elution of carbohydrates from silica gel showed that 95% of the sugars glucose, mannose, galactose, arabinose, xylose and sucrose are recovered. McKelvy and Scocca[77] obtained a 100% recovery of fucose, xylose and mannose from a layer of cellulose treated with tetrahydroborate. The degree of sugar recovery is not specified in the methods based on elution of the coloured products.

10. SUMMARY

Thin-layer chromatography, with its inherent simplicity of operation and sufficiently high degree of sensitivity and resolution, can, as a preliminary technique in the analysis of sugars and related compounds, replace other more sophisticated procedures such as gas–liquid and ion-exchange chromatography. Furthermore, as far as the identification of the sugar compounds is concerned, TLC is superior because it permits the use of rapid differential reactions on the layer and the utilization of other criteria such as R_F values and colour variations.

This technique has been widely used in studies of the carbohydrates and their derivatives in the biological and clinical fields. A wide range of monosaccharides, oligosaccharides and derivatives of monosaccharides can be separated on cellulose, silica gel and Kieselguhr layers. Many solvent systems, adaptible to each of these sorbents, have been reported. Two-dimensional separation of sugars and related compounds can be easily and rapidly performed. The samples are applied directly to the chromatographic layer without any pre-treatment. Quantitative determination of the sugars can be thus carried out with sufficient accuracy on crude analytical samples.

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Vol. 127, No. 2

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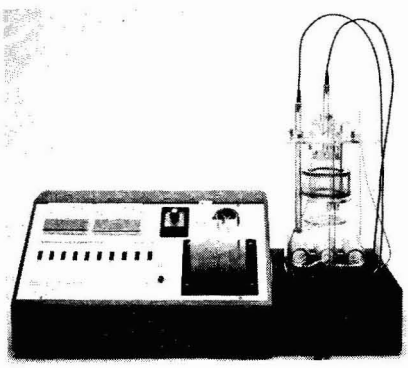
APPARATUS

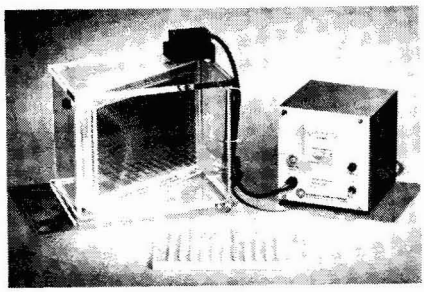
N-887

MACRO PREPARATIVE PGE SYSTEM

Birchover Instruments' macro preparative polyacrylamide gel electrophoresis system is based on Brownstone's method for the rapid separation of up to 1.5 g of proteins (including enzymes) or other biological compounds. The sample is separated on the cylindrical gel (6 or 9 cm diameter, 5 cm high) into bands which pass through the gel into a small-volume collection chamber. Fractions (typically 1–4 ml) are automatically collected at pre-set intervals in



clean buffer solution and pumped directly to a collection system. The system is automatically controlled by a high-voltage programmer unit which allows variations of sample collection time and of electrophoresis voltages so that optimum resolution can be obtained for slow and fast moving bands.



GEL DESTAINER

New from Gelman is a rapid destainer that removes excess stain from polyacrylamide gels (in slab or column form) in a few hours. The Rapid Destainer (product No. 51521) consists of a plexiglass solvent reservoir, an electrophoretic grid with stainless-steel electrodes, a safety interlocking cover, and its own 25-V power supply. Gel slabs are inserted into the electrophoretic grid for destaining. Gel columns are destained in the gel column template accessory, which holds up to 12 columns and is designed to fit inside the electrophoretic grid.

N-896

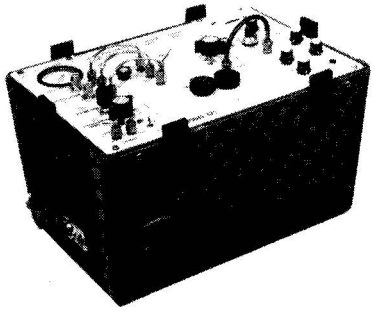
LC ACCESSORIES

Packard's LC accessories sheets Nos. 3501–3507 detail (with the aid of diagrams) the component parts (together with their ordering part numbers) of their LC columns and accessories. Sheet 3501 describes the 1/8-in. column, sheet 3502 the 1/4-in. column, and so on. Sheet 3507 deals with the glass column. A selection of books dealing with most areas of LC is given in LC accessories sheet 3509.

N-898

PORTABLE GAS CHROMATOGRAPH

The new Model 621 portable (21 lbs.) gas chromatograph from Analytical Instrument Development Inc. is available with four detector options (FID, ECD, FPD or TCD) all of which are capable of accepting a large number of valve options.



N-892

GC–MS ACCESSORIES

Available from Scientific Glass Engineering is a data sheet describing various GC–MS accessories, such as the low dead-volume micro needle valves (permitting leak-tight shut-off at 10^{-7} torr and pressures up to 1000 p.s.i.) which have been designed for fine control of critical capillary flows in GC–MS systems. The single-sidearm valves can be used, for example, as adjustable leak/isolation valves to MS. The dual-sidearm valves can be applied to infinitely variable effluent splitter/shut-off such as GC column to detector and MS.

Also described is the all-glass single-stage molecular jet separator for GC–MS interfacing. The construction allows accurate/reproducible jet sizes, alignment and spacing, and can be temperature cycled to 300°. A standard model is available for optimum input flows of 20–30 ml/min of helium, with an inlet jet of 0.1 mm, an outlet jet of 0.25 mm and jet spacing of 0.35 mm.

Further accessories (a low hold-up union, glass-lined metal tubing, GLT tee pieces, high vacuum connector, and graphite front ferrules) are also described.

N-904

ORGANIC VAPOUR MONITORING SYSTEM

Bendix Corporation has announced a new gas chromatograph (Model 2720), with a built-in flasher, for organic vapour monitoring. The flasher has been designed for the thermal desorption of concentrated vapour samples from a collection tube into the gas chromatograph which provides a complete analysis of the air sample, and the system has been found effective in monitoring organic vapours such as vinyl chloride, bis-chloromethyl ether, ketones. The gas chromatograph features a front opening door with a low mass oven for rapid programming and cool-down, as well as an optional linear temperature programmer.

N-901

UNIMETRICS CATALOGUE

Unimetrics announces a new 44-page catalogue giving details of their complete line of micro-syringes, micro-pipettes, valves, fittings and other chromatography accessories. Products include the modular micro-syringes with external metal scale, repeating dispensers for repetitive sample dispensing, and adjustable repeating syringes for pipetting in 2.5, 5.0 and 10.0 ml capacities. New products available are high-pressure sample injectors, rotary valves, pneumatic actuators, miniature tubing fittings, Teflon tubing, precision columns, and a new economical series of micro-pipettes.

CHEMICALS

N-889

NEW SUPELCO LIPID PUBLICATION

Supelco has recently published the first issue of the "Lipid Reporter" (replacing the Lipid Bulletin). Featured are Supelco natural phospholipids and triglycerides, and aqueous solutions of cholesterol, triolein and sphingomyelin for clinical and structural analysis.

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

NEW BOOKS

Rodd's chemistry of carbon compounds, 2nd ed., Vol. III, Aromatic compounds, Part D, edited by S. Coffey, Elsevier, Amsterdam, New York, 1976, xx + 324 pp., price US$ 53.95, Dfl. 140.00 (subscription price US$ 46.25, Dfl. 120.00), ISBN 0-444-41209-3.

Theory and practice of MO calculations in organic molecules (Progress in Theoretical Organic Chemistry, Vol. 1), by I.G. Csizmadia, Elsevier, Amsterdam, New York, 1976, x + 378 pp., price US$ 38.50, Dfl. 100.00, ISBN 0-444-41468-1.

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Adsorption at solid surfaces (Proceedings of the 2nd Interdisciplinary Surface Science Conference, University of Warwick, March 17–20, 1975; reprinted from *Surface Science*, Vol. 53), edited by C.R. Brundle and C.J. Todd, North-Holland, Amsterdam, New York, 1975, x + 746 pp., price US$ 86.50, Dfl. 225.00, ISBN 0-7204-0423-1 (Amsterdam), 0-444-11071-2 (New York).

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BASIC in chemistry: a self-instructional computing course, by G. Beech, Sigma Technical Press, Albrighton, 1976, 83 pp., ISBN 0-905104-00-5.

Chemistry and physics of carbon, Vol. 12, edited by Ph.L. Walker and P.A. Thrower, Marcel Dekker, New York, Basel, 1975, xi + 217 pp., price US$ 29.50, ISBN 0-8247-6304-1.

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Rules for the nomenclature of organic chemistry, Section E, Stereochemistry, (*Pure Appl. Chem.*, Vol. 45, No. 1), by L.C. Cross and W. Klyne, Pergamon, Oxford, 1976, price US$ 6.00, £ 3.00, ISBN 0-08-021019-8.

Spin–spin coupling and the conformational states of peptide systems, by V. Bystrow, Pergamon, Oxford, 1976, 81 pp., 27 figs., price US$ 12.50, £ 6.25, ISBN 0-08-019463-X.

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Analytical Isotachophoresis and Ion-Exchange Chromatography – A Comparison

Contact:
Mr. C.F. Simpson, School of Molecular Sciences, University of Sussex, Falmer, Brighton, Sussex BN1 9QJ, Great Britain

September 27–30, 1976
Szeged, Hungary

1st Danube Symposium on Chromatography

Contact:
Hungarian Chemical Socity, P.O. Box 240, H-1368 Budapest, Hungary. (Further details published in Vol 117, No. 1)

September 27–30, 1976
Brno, Czechoslovakia

International Microsymposium on Chromatography and Related Techniques

Contact:
Institute of Analytical Chemistry of the Czechoslovak Academy of Sciences, Leninova 82, 662 28 Brno, Czechoslovakia (Further details published in Vol. 118, No. 3)

November 1–4, 1976
Houston, Texas, U.S.A.

Chromatography '76. 11th International Symposium on Advances in Chromatography

Contact:
Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, Texas 77004, U.S.A. (Further details published in Vol. 117, No. 1)

February 28–March 4, 1977
Cleveland, Ohio, U.S.A.

28th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy

Contact:
John E. Graham, Program Chairman, Koppers Company, Inc., 440 College Park Drive, Monroeville, Pa. 15146, U.S.A.

April 26–28, 1977
Bratislava, Czechoslovakia

5th International Symposium. Improvements and Application of Chromatography in the Chemical Industry

Contact:
Ing. J. Remeň, Analytická sekcia ČS VTS, pri. n. p. Slovnaft, 82300 Bratislava, Czechoslovakia

May 22–27, 1977
Davos, Switzerland

International Symposium on Microchemical Techniques 1977

Contact:
Dr. W. Merz, BASF A.G. Untersuchungslaboratorium, WHU, D-6700 Ludwigshafen, G.F.R. (Further details published in Vol. 117, No. 1)

September 27–30, 1977
Salzburg, Austria

3rd International Symposium on Column Liquid Chromatography

Contact:
Professor Dr. J.K.F. Huber, Analytisches Institut der Universität Wien, Währinger Strasse 38, A-1090 Vienna, Austria

PUBLICATION SCHEDULE FOR 1976

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	D 1975	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	115/1 115/2	116/1 116/2	117/1 117/2	118/1 118/2	118/3 119	120/1 120/2	121/1 121/2	122 123/1	123/2	124/1 124/2 125/1	125/2 125/3	126 128/1	128/2 129
REVIEWS*					127/1				127/2			127/3	

* Volume 127 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.

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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.

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