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EDITORIAL

We should like to congratulate our editor of "Chromatographic Data", Dr. C. B. COULSON on his appointment to a chair at the University of Ghana and announce that he has consented to act as member of the editorial board for Africa from October 1963. We are also pleased to be able to welcome Dr. G. C. CASINOVI, who will join the *Journal of Chromatography* from the beginning of the new year as co-editor of "Chromatographic Data" so that at the same time this section will be expanded (probably from 8 to 16 pages) in order to maintain an adequate coverage of the literature.

As announced in August, this issue contains the first instalments of the expanded bibliography section which now encompasses Gas Chromatography and Thin-layer Chromatography as well as Paper Chromatography, their arrangement being discussed in detail by their respective editors.

A further increase in the number of papers submitted recently has caused us great concern as to its effect on an ever increasing backlog on publication dates and we hope to obviate this inconvenience by the publication of 300-page issues for the first four months of 1964 and then 150-page issues for the rest of the year. This measure taken together with the appearance of 4 volumes from next year on should have an appreciable and permanent effect on the waiting time between submission and publication of papers.

GAS–LIQUID CHROMATOGRAPHY IN QUALITATIVE ANALYSIS

PART V. THE DETERMINATION OF RELATIVE RETENTIONS IN R_{x9} AND RETENTION INDEX UNITS BY MEANS OF SECONDARY STANDARDS

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(Received January 28th, 1963)

INTRODUCTION

Recent proposals[1] have advocated the use of the *n*-alkanes as standards for the determination of relative retention data; the results being expressed in R_{x9}[2] and retention index units[3]. Though it is possible to use the *n*-alkanes as standards with practically all stationary phases it is often necessary to use other substances as secondary standards, particularly for the analysis of complex mixtures. Methods of calculating R_{x9} values and retention indices by means of secondary internal standards have therefore been investigated in an attempt to rationalise relative retention data.

EXPERIMENTAL

Chromatograms were obtained using an apparatus consisting of pyrex glass columns (5 ft. long, 4 mm I.D.) with a modified flame ionisation detector[4]. A mixture of hydrogen and nitrogen (3:1 by vol.) was used as carrier gas. This was dried over linde molecular sieve and was allowed to flow continuously in order to ensure reproducible retention data[5]. The column was packed with a 20% w/w mixture of polyethylene glycol-400 (Union Carbide) and acid washed (1% phosphoric acid) 60–70 mesh celite (JJ.'s, Ewell, Surrey). The column was heated by means of a water vapour jacket. Samples for analysis were introduced as dilute solutions in *n*-heptane (Phillips pure grade) by by means of stainless steel capillary pipettes. The solutes used were either obtained commercially or synthesised by colleagues at the Natural Rubber Producers' Research Association.

PROCEDURE

In order to calculate the retention of an unknown in R_{x9} or retention index units by means of a secondary internal standard the retention of the latter must be known in these units. These standards which may be either single substances or members of a homologous series may be calibrated directly or by means of the functional group retention parameters Functional Retention Index (F.R.I.[6]) or ΔMe[7].

The relative retentions of a number of chemically unrelated substances have been

determined using octan-2-ol, fatty acid methyl esters, and *n*-alkanes as internal standards in order to illustrate and to test the reliability of the proposed calibration procedures.

1. Determination of the R_{x9} values and retention indices with the n-alkanes as standards

The chromatogram of a convenient calibration series of *n*-alkanes (*n*-decane to *n*-nonadecane) was recorded and the retention distances measured from the point of injection to peak maxima on the recorder chart. The dynamic column dead volume was computed by linearisation of the "log plot"[8] and the R_{N9} values of the *n*-alkanes calculated by the slope of the "log plot" method[8].

Chromatograms of solutions containing one solute and two *n*-alkanes, eluted either side of the unknown, were then recorded and the retention distances measured. The mean R_{x9} value was calculated from the corrected retention distances using the expression:

$$R_{x9} = R_{xN} \times R_{N9} \tag{1}$$

where R_{xN} is the retention of the unknown relative to the *n*-alkane standard carbon number N and R_{N9} the retention of that *n*-alkane relative to *n*-nonane.

The retention indices of the solutes were calculated from the same corrected retention distances using the expression:

$$I = 100N + 100n \left\{ \frac{\log R_x - \log R_N}{\log R_{N+n} - \log R_N} \right\} \tag{2}$$

where N is the carbon number of the first *n*-alkane standard and $N + n$ that of the second,

R_x the retention distance of the unknown

R_N and R_{N+n} the retention distances of the *n*-alkanes carbon number N and $N + n$, respectively.

As far as possible consecutive *n*-alkanes were used.

2. Determination of the R_{x9} values and retention indices with octan-2-ol as standard

The chromatogram of a mixture of octan-2-ol and two *n*-alkanes, eluted either side of the alcohol, was recorded, the retention distances measured, and the retention index and R_{x9} value determined as described in the preceding section.

Chromatograms of mixtures of octan-2-ol and each of the solutes shown in Table II were then recorded in turn. The retention distances were measured and corrected for the column dead volume. The R_{x9} values and retention indices were calculated using the expressions:

$$R_{x9} = R_{xs} \times R_{s9} \tag{3}$$

where R_{xs} is the retention of the unknown relative to the secondary standard and R_{s9} the retention of the standard relative to *n*-nonane, and

$$I = 100 \left\{ \frac{\log R_{xs}}{b} + I_s' \right\} \tag{4}$$

where I_s' is the effective carbon number of the standard (retention index divided by

100) and b the slope of the n-alkane "log plot" which is assumed to remain constant. This can be obtained from the retentions of two n-alkanes using the expression:

$$b = \frac{\log R_{N+n} - \log R_N}{n} \tag{5}$$

where $N + n$ and N are the carbon numbers of the two n-alkanes.

Equation (3) is derived from eqn. (1) by simple algebra whilst eqn. (4) follows from the relation between retention index and R_{x9}[7].

3. Determination of the R_{x9} values and retention indices with the fatty acid methyl esters as standards

The retentions of the methyl esters were determined using:

(1) The n-alkanes as internal standards as described in Section 1.

(2) The functional group retention parameters ΔMe and F.R.I.

The mean ΔMe value for the methyl ester group was calculated from the R_{x9} values of the five homologues using the expressions:

$$Me = 14{\cdot}03 \left\{ \frac{\log R_{x9}}{b} \right\} + 128{\cdot}25 \tag{6}$$

and

$$\Delta Me = Me - M \tag{7}$$

where M is the molecular weight of the ester.

The mean F.R.I. value was determined from the retention indices by means of the expression:

$$F_{\mathrm{COOMe}} = I_{\mathrm{R \cdot COOMe}} - I_{\mathrm{R \cdot H}} \tag{8}$$

where $I_{\mathrm{R \cdot COOMe}}$ and $I_{\mathrm{R \cdot H}}$ are the retention indices of the methyl ester and n-alkane corresponding to the alkyl chain of the fatty acid.

In view of the close agreement of the results obtained by the two methods (see Table I) only those calculated using ΔMe and F.R.I. were used subsequently.

Chromatograms of solutions containing one unknown and two methyl esters, eluted on either side of the unknown, where then obtained, the retention distances measured and corrected for the column dead volume. The mean R_{x9} value of the unknown was calculated by means of eqn. (3) using the retentions of the two esters in turn.

The retention indices of the unknowns were obtained from the same retention distances using the expression:

$$I = 100N + F_{\mathrm{COOMe}} + 100n \left\{ \frac{\log R_x - \log R_1}{\log R_2 - \log R_1} \right\} \tag{9}$$

where N is the number of carbon atoms in the alkyl chain of the first methyl ester, n the difference in the carbon numbers of the two ester standards, and R_x, R_1 and R_2 the retention distances of the unknown and the two esters in order of increasing retention.

Though we in this case have used five homologues in order to determine the mean ΔMe and F.R.I. values in normal practice two homologues would be sufficient except for highly polar materials on polar stationary phases, for instance the n-alcohols see Table I.

DISCUSSION AND RESULTS

The greater part of the relative retention data in the literature has been determined using single substances as internal standards. Thus calibration of the standard by the methods described for octan-2-ol under the same column conditions should enable the conversion of the data into more widely acceptable units. The internal standard can be used for the concurrent determination of retention data and absolute concentrations. Though the *n*-alkanes can be used in this respect it is often preferable to use a standard of similar chemical character to the compounds being analysed; particularly when using capillary pipettes in order to minimise errors due to preferential volatilisation during injection. With thermally unstable stationary phases it should be appreciated that any change of the retentive character of the column will cause serious fluctuations of the retentions of the secondary standards relative to the *n*-alkanes. In such cases a different stationary phase should be used.

The errors involved in the direct calibration of a homologous series of secondary standards are minimised if they are chromatographed together with the *n*-alkanes. The calculation of the retentions by means of ΔMe or F.R.I. values requires that these parameters remain constant throughout a homologous series. Whereas the ΔMe values for six series of monosubstituted *n*-alkanes determined using dinonyl phthalate as stationary phase were found to be constant from *n*-propyl onwards[9], SWOBODA found that the F.R.I. values for the saturated aliphatic aldehydes increased with chain length from *n*-butanal to *n*-decanal.[9] Thus, it was necessary to check the constancy of these parameters with the polyethylene glycol stationary phase. The functional group retentions of the *n*-alcohols were found to increase from *n*-butanol to *n*-octanol presumably due to electronic interaction between the hydroxyl group and the adjacent methylene groups. The less polar methyl esters, however, remained constant from methyl *n*-butyrate as shown in Table I. Also included in this table are the observed R_{x9} and retention indices and those calculated by means of the functional group retention parameters. Clearly when the effective contribution of the functional group to the retention of a molecule varies throughout a series the observed and calculated retentions deviate seriously particularly with the lower homologues.

The procedure is capable of extension to any column temperature since ΔMe has been found to vary linearly with temperature according to the expression:

$$\Delta Me_\theta = \Delta Me_0 + \mu\theta \qquad (10)$$

where ΔMe_θ and ΔMe_0 are the values at $\theta°$ and 0° and μ the temperature coefficient[9]. Thus if ΔMe_0 and μ are known for a homologous series the retentions can be calculated in R_{x9} units at any column temperature for which b is known. F.R.I. should vary linearly for monosubstituted *n*-alkanes for which ΔMe and F.R.I. are simply related[10].

Since this method of predicting the retentions of the secondary standards is dependent upon the constancy of ΔMe and F.R.I. within a homologous series, it is important that the constancy should be established, particularly with strongly polar solutes on polar phases.

The relative retentions of a number of substances have been determined in R_{x9} and retention index units using the *n*-alkanes, methyl esters and octan-2-ol as standards by the methods described in the procedure section. The results (in Table II) reveal

TABLE I

THE FUNCTIONAL GROUP RETENTION PARAMETERS ΔMe AND F.R.I. FOR *n*-ALCOHOLS AND METHYL ESTERS AND COMPARISON OF RETENTIONS CALCULATED USING THESE PARAMETERS WITH OBSERVED DATA

Substance	*ΔMe*	*F.R.I.*	*Retention in R_{x_9} units*			*Retention in retention index units*		
			Observed	*Calculated**	*Percent Error*	*Observed*	*Calculated**	*δI***
Butan-1-ol	+ 106.28	871.8	7.33	8.20	11.9	1271.8	1293.0	+ 21.2
Pentan-1-ol	+ 107.76	882.4	13.25	14.03	5.9	1382.4	1393.0	+ 10.6
Hexan-1-ol	+ 108.49	887.6	23.27	23.93	2.8	1487.6	1493.0	+ 5.4
Heptan-1-ol	+ 109.06	891.5	40.62	40.83	0.5	1591.5	1593.0	+ 1.5
Octan-1-ol	+ 109.38	894.6	70.55	69.81	1.0	1694.6	1693.0	— 1.6
Methyl *n*-butyrate	+ 48.67	760.6	2.358	2.377	0.8	1060.6	1062.6	+ 2.0
Methyl *n*-hexoate	+ 48.90	762.3	6.92	6.93	0.1	1262.3	1262.6	+ 0.3
Methyl *n*-octanoate	+ 48.87	762.2	20.10	20.14	0.2	1462.2	1462.6	+ 0.4
Methyl *n*-nonanoate	+ 48.96	762.8	34.39	34.35	0.1	1562.8	1562.6	— 0.2
Methyl *n*-decanoate	+ 49.01	763.2	58.76	58.59	0.3	1663.2	1662.6	— 0.6
Methyl *n*-dodecanoate	+ 48.93	762.6	170.30	170.30	Nil	1862.6	1862.6	Nil

* Calculated using mean values of ΔMe = + 109.22 and F.R.I. = 893.0 for the *n*-alcohols and + 48.93 and 762.6 for the methyl esters.

** δI is the difference in observed and calculated retention indices.

TABLE II

RELATIVE RETENTIONS IN R_{x_9} AND RETENTION INDEX UNITS DETERMINED USING *n*-ALKANES, METHYL ESTERS AND OCTAN-2-ol AS STANDARDS

Substance	*Retention in R_{x_9} units*			*Retention in retention index units*		
	n-Alkanes	*Esters*	*Octan-2-ol*	*n-Alkanes*	*Esters*	*Octan-2-ol*
6-Methylhept-5-en-2-one	17.53	17.62	17.48	1436.5	1437.5	1436.0
Phenetole	19.59	19.76	19.56	1457.5	1459.0	1457.1
1-Bromononane	25.75	25.86	25.77	1508.6	1509.5	1508.8
1-Ethyl-2-methylpent-2-enol	30.23	30.28	—*	1538.6	1538.9	—*
Diallyl disulphide	31.64	31.79	—*	1547.0	1548.1	—*
1,1-Dimethylbutyl-2-methyl pent-2-enyl sulphide	36.92	37.10	36.89	1575.9	1577.0	1575.9
Di-isopropyl trisulphide	38.30	38.35	38.18	1583.0	1583.3	1582.7
n-Decylaldehyde	40.20	40.40	40.16	1592.1	1593.0	1591.7
N,N-Methyl-ethyl-aniline	58.48	58.88	58.55	1662.5	1663.5	1662.5
Di-(2-methylpent-2-enyl)sulphide	66.94	67.48	66.85	1687.7	1688.7	1687.4
N,N-Diethylaniline	67.61	67.59	67.30	1689.5	1689.4	1688.6
Percentage standard deviation	—	0.53	0.23	—	—	—
Standard deviation in retention index units	—	—	—	—	0.9	0.4

* Not resolved from octan-2-ol peak.

It is normal practice to express as a percentage standard deviation for R_{x_9} and as a standard deviation in retention index units for retention index.

an excellent agreement between the values obtained using the various standards particularly as only one *n*-alkane calibration was recorded; the value of b being assumed to remain constant during the period of the investigation (approximately 5 days). Recent work[11] using vapour jacket heating has shown that fluctuations of barometric pressure have a small but significant effect on b and R_{x9} and to a lesser extent retention index. Therefore one would expect an even better agreement between the retentions obtained using the different standards with good electrical heating where a control of better than $\pm$ 0.1° is possible.

ACKNOWLEDGEMENT

The author thanks Dr. J. F. SMITH AND Mr. G. M. C. HIGGINS for helpful discussions during the preparation of this paper.

SUMMARY

Procedures are described whereby relative retentions can be determined in R_{x9} and retention index units by means of internal standards other than *n*-alkanes. Such procedures are important as they enable the calculation of all relative retention data in either of two simply related units.

REFERENCES

1 M. B. EVANS, G. M. C. HIGGINS AND J. F. SMITH, to be published.
2 J. F. SMITH, *Chem. Ind. (London)*, (1960) 1024.
3 E. KOVATS, *Helv. Chim. Acta*, 41 (1958) 1915.
4 J. F. SMITH, in R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, London, 1960, p. 114.
5 M. B. EVANS AND J. F. SMITH, to be published as Part VI.
6 P. A. T. SWOBODA, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962, p. XXX.
7 M. B. EVANS AND J. F. SMITH, *Nature*, 190 (1961) 905.
8 M. B. EVANS AND J. F. SMITH, *J. Chromatog.*, 9 (1962) 147.
9 M. B. EVANS AND J. F. SMITH, *J. Chromatog.*, 8 (1962) 303.
10 P. A. T. SWOBODA, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962, p. 282.
11 M. B. EVANS, to be published.

ISOTOPIC EFFECTS IN GAS-LIQUID CHROMATOGRAPHY

I. GAS CHROMATOGRAPHIC BEHAVIOUR OF BENZENE AND PERDEUTEROBENZENE

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The possibility of using gas chromatography for the separation of natural isotopes has been investigated so far only for hydrogen[1-4] and neon[5]. The introduction of high efficiency columns, however, opens new possibilities for the determination of isotopic molecules. A partial separation of deuterated and tritiated cyclohexane has been obtained by WILZBACH AND RIESZ[6], but more recently FALCONER AND CVETANOVIC[7] have shown that a number of hydrocarbons can be effectively separated from their partially and fully deuterated isomers on capillary columns.

The purpose of this investigation was to evaluate the factors which affect the separation and the determination of two isotopically substituted molecules by gas-liquid chromatography with the aim of obtaining a number of fundamental data, which may then be extended to the analysis of any system. This work deals with the gas chromatographic investigation of benzene and perdeuterobenzene where the mass difference is about 7.7 %.

EXPERIMENTAL

A home-made gas chromatograph was used with a hydrogen flame ionization detector; the main advantage is, in comparison to commercially available instruments, that it can be thermostated by a circulating liquid so that an accuracy of about 0.1 °C can easily be obtained in the temperature range 0-50°.

Perdeuterobenzene was obtained from Fluka (Buchs); its purity was higher than 99 %.

Experiments were performed to estimate the response of the hydrogen flame detector towards benzene and perdeuterobenzene. It was found that the ratio of the peak areas is the same as the ratio of the molar concentrations in the range of the experimental error (1-2 %).

Glass capillary columns, 0.3 mm internal diameter were used, and prepared as described by DESTY *et al.*[8]. The experiments were carried out on columns coated, as described elsewhere[9], with the following liquid phases: dinonyl phthalate (A), squalane (B) and silicone oil 702 (C). The number of theoretical plates and the length of these columns, (taking *n*-heptane as internal standard), were: A 40,000 (40 m long); B 40,000 (40 m long); C 160,000 (80 m long).

Retention volumes have been corrected for the dead volume according to PETERSON AND HIRSH[10].

The gas chromatograms of benzene and perdeuterobenzene, performed on these columns under the same experimental conditions, are shown in Fig. 1. The ratio of the retention volumes is 1.0095 on A, 1.0357 on B and 1.0198 on C.

The nature of the stationary phase plays an important role in the separation of these substances. From Fig. 1 it appears that on a less polar phase *e.g.* squalane, better results are obtained, only a barely appreciable separation being observed on the dinonyl phthalate column.

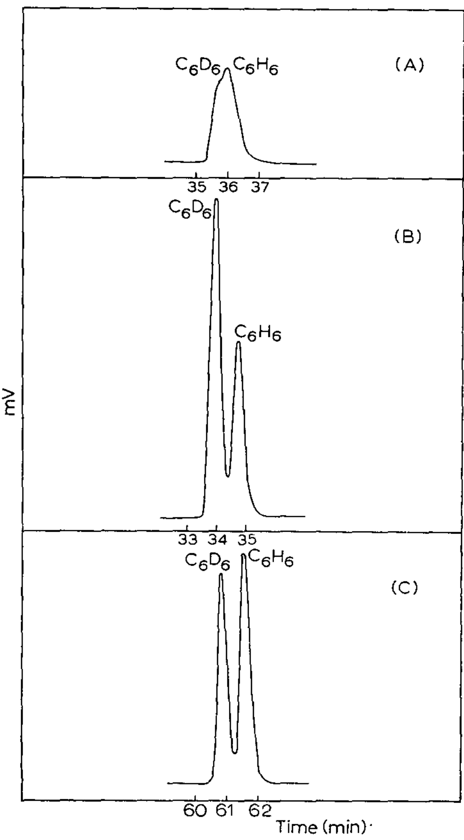

Fig. 1. Gas chromatograms of benzene and perdeuterobenzene on capillary columns coated with dinonyl phthalate (A: 40 m), squalane (B: 40 m) and silicone oil (C: 80 m). Temperature: 20°; carrier gas: nitrogen; flow rate: 0.5 ml/min.

In order to make a quantitative appraisal of this behaviour, the thermodynamics of the chromatographic process were studied. The results were calculated not in terms of the absolute values but as the differences between the changes of the thermodynamic functions. It should be pointed out that absolute retention volumes can be measured only with an accuracy of about 3 %. Calculations on the ratio of relative retention volumes obtained by running a combined sample afford a degree of accuracy about one hundred times higher. Gas chromatograms of benzene (Bz) and perdeuterobenzene (dBz) were run at different temperatures on the squalane and silicone oil columns and the ratio of the corrected retention volumes: ($V°_R$ Bz/$V°_R$ dBz), is

TABLE I

T	$\frac{P_{dBz}}{P_{Bz}}$	$\log \frac{P_{dBz}}{P_{Bz}} \times 10^3$	Squalane				Silicone oil 702			
			$\frac{V_R{}^\circ{}_{Bz}}{V_R{}^\circ{}_{dBz}}$	$\log \frac{V_R{}^\circ{}_{Bz}}{V_R{}^\circ{}_{dBz}} \times 10^3$	$\frac{\gamma_{dBz}}{\gamma_{Bz}}$	$\log \frac{\gamma_{dBz}}{\gamma_{Bz}} \times 10^3$	$\frac{V_R{}^\circ{}_{Bz}}{V_R{}^\circ{}_{dBz}}$	$\log \frac{V_R{}^\circ{}_{Bz}}{V_R{}^\circ{}_{dBz}} \times 10^3$	$\frac{\gamma_{dBz}}{\gamma_{Bz}}$	$\log \frac{\gamma_{dBz}}{\gamma_{Bz}} \times 10^3$
273.2	1.0340	14.520	1.0421	17.909	1.0073	3.1157	—	—	—	—
283.2	1.0332	14.142	1.0395	16.824	1.0061	2.6411	1.0205	8.8130	0.9880	—5.24
293.2	1.0315	13.469	1.0357	15.234	1.0041	1.7770	1.0198	8.5150	0.9887	—4.94
303.2	1.0304	13.006	1.0336	14.352	1.0031	1.3442	1.0191	8.2168	0.9890	—4.80
313.2	1.0295	12.626	—	—	—	—	1.0186	8.0037	0.9894	—4.63
323.2	1.0286	12.240	—	—	—	—	1.0178	7.6624	0.9895	—4.58

compared in Table I with the corresponding ratio of the vapour pressures, (PdBz/PBz) measured by INGOLD *et al.*[11]. With the squalane column the former ratio is higher than the latter. The converse is observed with the silicone oil column. The interaction of the two molecules is therefore quite different with the two liquid phases.

In Fig. 2 the logarithm of the ratio of the retention volumes on squalane column

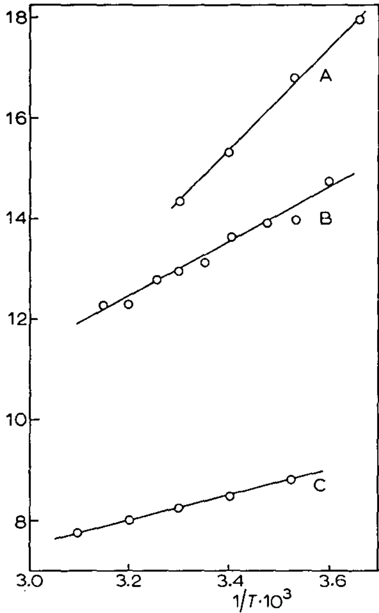

Fig. 2. Logarithm × 10^3 of the ratio of the retention volumes (benzene/perdeuterobenzene) on squalane (A) and silicone oil (C) and of the vapour pressures (perdeuterobenzene/benzene) *versus* $1/T$ (B).

(A) and on silicone oil column (C) and the logarithm of the ratio of the vapour pressures (B)[6] is plotted against the reciprocal of the absolute temperature. There is a linear relationship between these values, which holds in the range of temperature investigated. It can be expressed by the following equation:

$$\log \frac{V^{\circ}_{R}\,\mathrm{Bz}}{V^{\circ}_{R}\,\mathrm{dBz}} = -\frac{(\Delta H_{\mathrm{Bz}} - \Delta H_{\mathrm{dBz}})_{\mathrm{chr.}}}{2.3\,R} \cdot \frac{1}{T} + C \qquad (1)$$

From the slopes of A and C on the graph the difference of enthalpy related to the chromatographic process can be calculated; the results are:

on squalane: $(\Delta H_{\mathrm{Bz}} - \Delta H_{\mathrm{dBz}})_{\mathrm{chr.}} = -46.2$ cal.

on silicone oil: $(\Delta H_{\mathrm{Bz}} - \Delta H_{\mathrm{dBz}})_{\mathrm{chr.}} = -11.4$ cal.

By extrapolation to $1/T = 0$, the value of the constant C is determined and from it the difference of the entropy change calculated by the following equation:

$$\Delta S_{\mathrm{Bz}} - \Delta S_{\mathrm{dBz}} = 2.3\,RC \qquad (2)$$

The difference of entropy can also be calculated from the following relationship:

$$\Delta G_{\mathrm{Bz}} - \Delta G_{\mathrm{dBz}} = \Delta H_{\mathrm{Bz}} - \Delta H_{\mathrm{dBz}} - T(\Delta S_{\mathrm{Bz}} - \Delta S_{\mathrm{dBz}})_{\mathrm{chr.}}$$

The differences of free energy changes are obtained from the retention volumes:

$$\Delta G_{\mathrm{Bz}} - \Delta G_{\mathrm{dBz}} = -2.3\ RT \log \frac{V^{\circ}_{R\,\mathrm{Bz}}}{V^{\circ}_{R\,\mathrm{dBz}}}$$

Both values are given in Table II.

TABLE II

T	$(\Delta S_{Bz}-\Delta S_{dBz})_c \times 10^2$	Squalane			Silicone oil 702		
		$\Delta G_{Bz}-\Delta G_{dBz}$	$(\Delta S_{Bz}-\Delta S_{dBz})_{chr} \times 10^2$	$(\Delta S_{Bz}-\Delta S_{dBz})_m \times 10^2$	$\Delta G_{Bz}-\Delta G_{dBz}$	$(\Delta S_{Bz}-\Delta S_{dBz})_{chr} \times 10^2$	$(\Delta S_{Bz}-\Delta S_{dBz})_m \times 10^2$
273	—7.69	—22.31	—8.75	—1.06	—	–	—
283	—7.42	—21.73	—8.65	—1.23	—11.38	0	7.42
293	—7.17	—20.37	—8.82	—1.65	—11.38	0	7.17
303	—6.93	—19.84	—8.70	—1.77	—11.36	0	6.93
313	—6.71	—	—	—	—11.43	0	6.71
323	—6.50	—	—	—	—11.29	0	6.50

The gas chromatographic process might be taken as consisting of two reactions, one due to condensation (c) of the eluted component and the other due to mixing (m) with the partition liquid. The calculated enthalpy and entropy changes are therefore the sum of the contributions due to each reaction:

$$\begin{aligned} (\Delta H_{\mathrm{Bz}} - \Delta H_{\mathrm{dBz}})_{\mathrm{chr.}} &= (\Delta H_{\mathrm{c\,Bz}} + \Delta H_{\mathrm{m\,Bz}}) - (\Delta H_{\mathrm{c\,dBz}} + \Delta H_{\mathrm{m\,dBz}}) \\ (\Delta S_{\mathrm{Bz}} - \Delta S_{\mathrm{dBz}})_{\mathrm{chr.}} &= (\Delta S_{\mathrm{c\,Bz}} + \Delta S_{\mathrm{m\,Bz}}) - (\Delta S_{\mathrm{c\,dBz}} + \Delta S_{\mathrm{m\,dBz}}) \end{aligned} \qquad (3)$$

By using the difference between the latent heat of these molecules determined by INGOLD *et al.* ($\Delta H_{\mathrm{c\,Bz}} - \Delta H_{\mathrm{c\,dBz}} = -20.96$ cal)[11], the difference of the changes of heat of mixing in excess is calculated as:

$$\Delta H_{\mathrm{m\,Bz}} - \Delta H_{\mathrm{m\,dBz}} = -25.2 \text{ cal for squalane}$$

and:

$$\Delta H_{\mathrm{m\,Bz}} - \Delta H_{\mathrm{m\,dBz}} = 9.6 \text{ cal for silicone oil.}$$

The values of entropy change of the process of mixing are calculated according to:

$$(\Delta S_{\mathrm{Bz}} - \Delta S_{\mathrm{dBz}})_{\mathrm{m}} = (\Delta S_{\mathrm{Bz}} - \Delta S_{\mathrm{dBz}})_{\mathrm{chr.}} - (\Delta S_{\mathrm{Bz}} - \Delta S_{\mathrm{dBz}})_{\mathrm{c}}$$

By considering the Clausius-Clapeyron equation for the vaporization of benzene and perdeuterobenzene

$$\log \frac{P^{\circ}_{\mathrm{dBz}}}{P^{\circ}_{\mathrm{Bz}}} = -\frac{\Delta H_{\mathrm{v\,dBz}} - \Delta H_{\mathrm{v\,Bz}}}{2.3\,R} \cdot \frac{1}{T} + C' \qquad (4)$$

and for the chromatographic process of these compounds (eqn. 1) and by introducing

the relationship[12]

$$\log \frac{V^{\circ}_{R\,\mathrm{Bz}}}{V^{\circ}_{R\,\mathrm{dBz}}} = \log \frac{(\gamma P^{\circ})_{\mathrm{dBz}}}{(\gamma P^{\circ})_{\mathrm{Bz}}} \tag{5}$$

where γ is the activity coefficient, and by combining eqns. (1), (4) and (5) and accounting for eqn. (3), the following equation is obtained, being $\Delta H_{\mathrm{v}} = -\Delta H_{\mathrm{c}}$:

$$\log \frac{\gamma_{\mathrm{dBz}}}{\gamma_{\mathrm{Bz}}} = -\frac{\Delta H_{\mathrm{mBz}} - \Delta H_{\mathrm{m\,dBz}}}{2.3\,R}\,\frac{1}{T} + (C' - C) \tag{6}$$

From the plot of the logarithm of the ratio of the activity coefficients, calculated according to eqn. (5), *versus* $1/T$ (Fig. 3), the difference between the integration constants

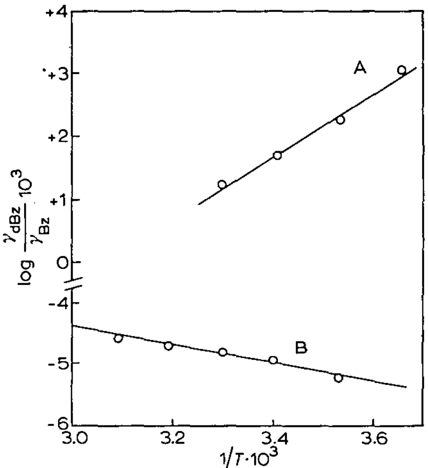

Fig. 3. Logarithm of the ratio of the activity coefficients (perdeuterobenzene/benzene) *versus* $1/T$.

is calculated by graphic extrapolation. From this the difference between the heats of mixing in excess is obtained. The values — 20.50 cal for squalane and + 6.4 cal for silicone oil are in fair agreement with the ones previously reported.

DISCUSSION

The data obtained from the gas chromatographic behaviour of benzene and perdeuterobenzene, given in Table I, show that the ratio of the activity coefficients in squalane is greater than one and in silicone oil less than one. This means that in squalane the difference between the heats of solution acts in the same direction as that of the heat of mixing, while the converse holds for silicone oil. Both ratios tend to unity on raising the temperature.

As a tentative explanation for this behaviour, the fact that in the liquid state there is a stronger attraction among the benzene molecules than is the case in perdeuterobenzene might be taken into account. These attractions, due to the interaction of π electrons in the two molecules, are responsible for the different vapour pressures of the two molecules. The dispersion strengths, due to the dipoles of a polar

phase, exert a strong competitive action against the association strengths with the result that on a polar stationary phase the two substances behave almost in the same way and therefore a lower separation is achieved.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to CNEN (Comitato Nazionale Energia Nucleare) which provided a grant for this research and to Prof. V. CAGLIOTI for his interest and helpful discussion.

SUMMARY

The separation of benzene and perdeuterobenzene by gas–liquid chromatography has been investigated on glass capillary columns. The nature of the liquid phase plays a very important role in the separation of isotopically substituted molecules, the less polar (squalane) being more effective than silicone oil. With the former, the separation is better than expected on account of the differences of vapour pressure, the reverse is found with the latter phase.

The thermodynamics of the chromatographic process have been studied, and the difference of ΔG, ΔH and ΔS for both substances together with the ratio of the activity coefficients on squalane and silicone oil have been calculated.

REFERENCES

1 W. R. MOORE AND W. R. WARD, *J. Am. Chem. Soc.*, 80 (1958) 2909.
2 C. O. THOMAS AND H. A. SMITH, *J. Phys. Chem.*, 63 (1959) 427.
3 S. OHKOSHI, Y. FUGITA AND T. KWAN, *Bull. Chem. Soc. Japan*, 31 (1958) 770.
4 M. MOHNKE AND W. SAFFERT, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962.
5 E. GLUECKAUF, K. H. BARKER AND G. P. KITT, *Discussions Faraday Soc.*, 7 (1949) 199.
6 K. E. WILZBACH AND P. RIESZ, *Science*, 126 (1957) 748.
7 W. E. FALCONER AND R. J. CVETANOVIC, *Anal. Chem.*, 34 (1962) 1064.
8 D. H. DESTY, J. N. HASEMAPE AND B. H. F. WHYMAN, *Anal. Chem.*, 32 (1960) 302.
9 F. BRUNER, G. CARTONI AND A. LIBERTI, *Chim. Ind. (Milan)*, 44 (1962) 999.
10 D. L. PETERSON AND J. HIRSH, *J. Lipid Res.*, 1 (1959) 32.
11 C. K. INGOLD, G. G. RAISIN, C. L. WILSON AND C. R. BAYLEY, *J. Chem. Soc.*, (1936) 915.
12 A. I. M. KEULEMANS, *Gas Chromatography*, Reinhold Publishing Corp., New York, p. 16.

COMPOUND IDENTIFICATION BY GAS CHROMATOGRAPHY

A COMPARISON TO THE PAPER CHROMATOGRAPHY METHOD OF SCHAUER AND BULIRSCH

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INTRODUCTION

Structural analysis by paper chromatography, first described by SCHAUER AND BULIRSCH[1] and discussed by MELOAN AND KISER[2], is potentially a very powerful technique for compound identification. The basic concept is that each individual group within a molecule contributes to the R_F value and that when these individual groups are combined into a molecule there is a further contribution to the R_F which is called a group constant. This group constant and the contributions per functional group are determined by solving mathematical equations involving R_F values. By using two different solvents to elute the components of two separate paper chromatograms it is possible to determine the number of each of two different functional groups within a compound such as the CH_2 and COOH of an acid. In order to determine three groups, three different solvents are necessary, for four groups, four solvents, etc.

The problem here was to see if the retention parameters of gas chromatography could be incorporated into the equations developed by SCHAUER AND BULIRSCH. Although qualitative gas chromatographic structural analysis has been tried by many others, MERRIT AND WALSH[3] for example, there seems to have been no attempt to correlate it to the above mentioned work.

EXPERIMENTAL

Instrumentation

The instruments used were an F and M 609 equipped with a hydrogen flame ionization detector and the second instrument was constructed in this laboratory[4], and employed 2000 Ω thermistor detectors.

Chemicals

Methylamine, propylamine. ethylenediamine, 1,4-butanediamine dihydrochloride and 1,6-hexanediamine were obtained from Eastman; 1,5-pentanediamine from K and K Laboratories, *n*-butylamine from Union Carbide and Carbon Co., and *n*-hexylamine from the Matheson Company.

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Those amines obtained as water solutions were dried over potassium hydroxide pellets. In the case of methylamine and ethylamine, the vials were covered with septum caps obtained from Fisher Scientific Company and a Yale syringe was used for sampling and injecting the gases. Since 1,6-hexanediamine solidified when the water was removed, the vial was warmed until the amine melted and a warmed syringe was used to inject the sample. The 1,4-butanediamine was liberated from its hydrochloride salt by mixing it with some potassium hydroxide pellets and adding a few drops of water.

Column materials and preparation

The columns were 6 ft. long and made of 1/4 in. O.D. aluminum tubing. Gas-Chrom Z was used as the solid phase in all of the columns. The liquid phases studied were Apiezon M Grease, Dow-Corning Silicone Oil 550, and Silicone Gum Rubber.

Column conditions

The retention times of the amines, Table I, were obtained at a column temperature of 95°, an injection port and detector block temperature of 110°, an inlet pressure of 7.5 p.s.i.g. and the exit at atmospheric pressure.

RESULTS AND DISCUSSION

Calculations

The "R_F" values were calculated in a manner similar to that for paper chromatography. The solvent front was taken to be the air peak and thus the movement of the solvent front is equal to the column length. The movement of the component in the column when the air peak emerges is given by:

$$\text{Movement of solute} = t_{air}/t_R \times \text{length of column.}$$

Using these definitions the "R_F" values for gas chromatography were obtained from the relation:

$$``R_F" = \frac{\text{movement of solute}}{\text{movement of solvent front}} = \frac{t_{air}/t_R \times \text{length of column}}{\text{length of column}} = t_{air}'/t_R.$$

Then according to SCHAUER AND BULIRSCH:

$$R_M = -\log (1/R_F - 1) = \log R_F/(1 - R_F).$$

And for gas chromatography using:

$$``R_M" = -\log (1/``R_F" - 1).$$

Plotting "R_M" *versus* the number of CH_2 groups for each column is shown in Figs. 1–3.

Next the matrix calculations were done for the three possible pairings of the three columns. In each case the knowns were taken to be *n*-propylamine, *n*-hexylamine, and 1,4-butanediamine. The *n*-pentylamine was used as the unknown. The calculations for the Apiezon M–Silicone Gum Rubber combination follow.

TABLE I

VALUES OF "R_F" AND "R_M" FOR AMINES ON DIFFERENT COLUMN LIQUID PHASES

No.	Compound	*20 % Apiezon M*			*20 % Silicone Gum Rubber*			*20 % Silicone Oil 550*		
		t_R	"R_F"	"R_M"	t_R	"R_F"	"R_M"	t_R	"R_F"	"R_M"
	Air	0.68			0.68			0.70		
1	Methylamine	0.96	0.708	+0.387	0.88	0.722	+0.530	0.83	0.844	+0.733
2	Ethylamine	1.25	0.544	+0.076	0.93	0.730	+0.432	0.90	0.777	+0.541
3	*n*-Propylamine	1.43	0.475	−0.046	1.18	0.575	+0.131	0.98	0.715	+0.398
4	*n*-Butylamine	2.18	0.312	−0.344	1.63	0.417	−0.146	1.22	0.573	+0.128
5	*n*-Pentylamine	3.35	0.203	−0.594	2.43	0.280	−0.410	1.58	0.443	−0.100
6	*n*-Hexylamine	6.08	0.112	−0.900	4.10	0.166	−0.702	2.32	0.302	−0.365
7	Ethylenediamine	1.98	0.343	−0.283	1.90	0.353	−0.253	1.75	0.400	−0.176
8	1,4-Butanediamine	6.38	0.107	−0.992	4.82	0.141	−0.785	3.84	0.182	−0.654
9	1,5-Pentanediamine	13.15	0.0517	−1.264	9.30	0.073	−1.104	6.65	0.105	−0.931
10	1,6-Hexanediamine	26.80	0.0254	−1.584	16.60	0.041	−1.370	11.95	0.0585	−1.207

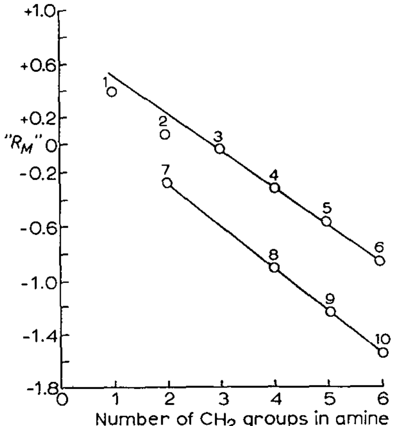

Fig. 1. Linear relation between "R_F" and the number of CH_2 groups in a molecule after transformation into "R_M" = — log (1/"R_F" — 1). The column was 6 ft. long and contained Apiezon M Grease on Gas-Chrom Z. The points represent the amines having the corresponding numbers in Table I, and the "R_M" values are those given in the table for the above column.

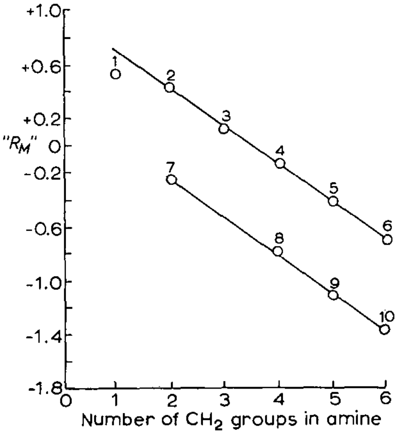

Fig. 2. Linear relation between "R_F" and the number of CH_2 groups in a molecule after transformation into "R_M" = — log (1/"R_F" — 1). The column was 6 ft. long and contained Silicone Gum Rubber on Gas-Chrom Z. The points represent the amines having the corresponding numbers in Table I, and the "R_M" values are those given in the table for the above column.

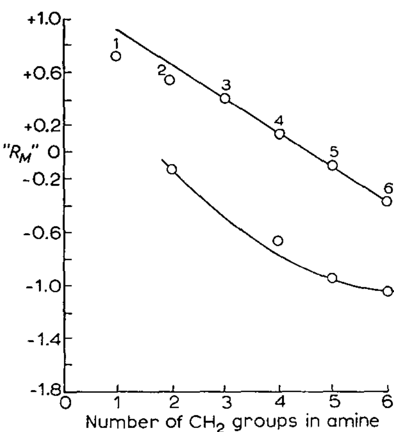

Fig. 3. Linear relation between "R_F" and the number of CH_2 groups in a molecule after transformation into "R_M" = — log (1/"R_F" — 1). The column was 6 ft. long and contained Dow-Corning Silicone Oil 550 on Gas-Chrom Z. The points represent the amines having the corresponding numbers in Table I, and the "R_M" values are those given in the table for the above column.

Test substance	*Apiezon M "$R_M{}^1$"*	*Silicone Rubber "$R_M{}^2$"*	*No. of groups*	
			CH_2	*NH_2*
Propylamine	—0.046	+0.131	3	1
Hexylamine	—0.900	—0.702	6	1
1,4-Butanediamine	—0.922	—0.785	4	2
Unknown	—0.594	—0.410	5	1

The determinant for this is:

$$\begin{pmatrix} Y_{\mathrm{CH_2}} \\ Y_{\mathrm{NH}} \end{pmatrix} = \frac{1}{\begin{vmatrix} -0.046 & +0.131 & 1 \\ -0.900 & -0.702 & 1 \\ -0.922 & -0.785 & 1 \end{vmatrix}} \times \begin{pmatrix} 3 \\ 1 \end{pmatrix} \begin{vmatrix} \text{"}R_M{}^1\text{"} & \text{"}R_M{}^2\text{"} & 1 \\ -0.900 & -0.702 & 1 \\ -0.922 & -0.785 & 1 \end{vmatrix}$$

$$+ \begin{pmatrix} 6 \\ 1 \end{pmatrix} \begin{vmatrix} \text{"}R_M{}^1\text{"} & \text{"}R_M{}^2\text{"} & 1 \\ -0.922 & -0.785 & 1 \\ -0.046 & +0.131 & 1 \end{vmatrix} + \begin{pmatrix} 4 \\ 2 \end{pmatrix} \begin{vmatrix} \text{"}R_M{}^1\text{"} & \text{"}R_M{}^2\text{"} & 1 \\ -0.046 & +0.131 & 1 \\ -0.900 & -0.702 & 1 \end{vmatrix}$$

The solutions for each terms are as follows, using the standard sign convention for matrices, +, —, +:

The denominator:

$$-0.046 \begin{vmatrix} -0.702 & 1 \\ -0.785 & 1 \end{vmatrix} +0.900 \begin{vmatrix} +0.131 & 1 \\ -0.785 & 1 \end{vmatrix} -0.922 \begin{vmatrix} +0.131 & 1 \\ -0.702 & 1 \end{vmatrix}$$

$$= -0.046\ (+0.083)\ +0.900\ (+0.916)\ -0.922\ (+0.833)$$

$$= -0.004 +0.825 -0.768 = +0.053.$$

The first term:

$$\text{"}R_M{}^1\text{"} \begin{vmatrix} -0.702 & 1 \\ -0.785 & 1 \end{vmatrix} +0.900 \begin{vmatrix} \text{"}R_M{}^2\text{"} & 1 \\ -0.785 & 1 \end{vmatrix} -0.922 \begin{vmatrix} \text{"}R_M{}^2\text{"} & 1 \\ -0.702 & 1 \end{vmatrix}$$

$$= +0.083\ \text{"}R_M{}^1\text{"} -0.022\ \text{"}R_M{}^2\text{"} +0.061.$$

The second term:

$$\text{"}R_M{}^1\text{"} \begin{vmatrix} -0.785 & 1 \\ +0.131 & 1 \end{vmatrix} +0.922 \begin{vmatrix} \text{"}R_M{}^2\text{"} & 1 \\ +0.131 & 1 \end{vmatrix} -0.046 \begin{vmatrix} \text{"}R_M{}^2\text{"} & 1 \\ -0.785 & 1 \end{vmatrix}$$

$$= -0.916\ \text{"}R_M{}^1\text{"} +0.876\ \text{"}R_M{}^2\text{"} -0.157.$$

The third term:

$$\text{"}R_M{}^1\text{"} \begin{vmatrix} +0.131 & 1 \\ -0.702 & 1 \end{vmatrix} +0.046 \begin{vmatrix} \text{"}R_M{}^2\text{"} & 1 \\ -0.702 & 1 \end{vmatrix} -0.900 \begin{vmatrix} \text{"}R_M{}^2\text{"} & 1 \\ +0.131 & 1 \end{vmatrix}$$

$$= +0.833\ \text{"}R_M{}^1\text{"} -0.845\ \text{"}R_M{}^2\text{"} +0.150.$$

In order to solve for the number of CH_2 groups present the first term was multiplied by 3, the second term by 6 and the third term by 4. Adding the results together gave:

$$-1.915\ \text{"}R_M{}^1\text{"} + 1.774\ \text{"}R_M{}^2\text{"} -0.159.$$

Dividing this by the denominator value of +0.053 gave:

$$-36.1 \text{ "}R_M{}^1\text{"} + 33.5 \text{ "}R_M{}^2\text{"} - 3.00.$$

Substituting the values of "$R_M{}^1$" and "$R_M{}^2$" of the unknown into this gave 4.7 for the number of CH_2 groups. This is nearest to 5, and thus the number of CH_2 groups in the unknown is calculated to be 5. To solve for the number of NH_2 groups the first term was multiplied by 1, the second term by 1 and the third term by 2. Adding the results together gave:

$$+0.833 \text{ "}R_M{}^1\text{"} - 0.854 \text{ "}R_M{}^2\text{"} + 0.204.$$

Dividing this by the denominator value of +0.053 gave:

$$+15.71 \text{ "}R_M{}^1\text{"} - 16.10 \text{ "}R_M{}^2\text{"} + 3.85.$$

Substituting the values of "$R_M{}^1$" and "$R_M{}^2$" of the unknown into this gave 1.1 for the number of NH_2 groups. This is nearest to 1, and thus the number of NH_2 groups in the unknown is calculated to be 1.

Thus, for this combination of columns the compound is correctly identified as pentylamine, $C_5H_{11}NH_2$. The results for the three column combinations are given in Table II. Notice that the results are not valid when a column is used which has a non-linear plot of "R_M" *versus* number of groups. This is the case with column 3 and neither set of results are correct when this column was used. It was found that if a least squares treatment was made to obtain a straight line that all of the values involved were changed too much to give accurate results.

TABLE II

RESULTS OF THE MATRIX CALCULATIONS OF THE NUMBER OF CH_2 AND NH_2 GROUPS IN THE UNKNOWN, PENTYLAMINE

Column No. combinations*	Calculated		Actual	
	Y_{CH_2}	Y_{NH_2}	Y_{CH_2}	Y_{NH_2}
1 and 2	+4.7	+1.1	5	1
1 and 3	+4.8	−1.3	5	1
2 and 3	−0.9	−0.9	5	1

* For column No. 1 the liquid phase is Apiezon M, for No. 2: Silicone Gum Rubber, and for No. 3: D-C Silicone Oil 550.

With each sample a small amount of air was intentionally injected so that the reference air peak could be used to locate the "solvent front". Since the amines used covered such a wide range of boiling points and molecular weights, not all of the peaks were of satisfactory shape for quantitative analysis purposes, however, satisfactory retention time data was readily obtained. The variation of the points for methylamine and ethylamine from a straight line in Figs. 1–3 is common for the lower members of a homologous series in gas chromatography[5].

In general, the plots of "R_M" *versus* the number of CH_2 groups (Figs. 1–3) give straight lines similar to paper chromatographic results. The slope of the lines are of

opposite sign to those obtained by SCHAUER AND BULIRSCH, due to the organic liquid being a part of the stationary phase in gas chromatography whereas it is the mobile phase in paper chromatography.

It can be seen that an analogy between paper chromatography and gas chromatography does exist for this "structure determination", however, the calculations are rather tedious for the obtaining of the final equation into which the "R_M" values from two columns for the unknown are substituted. Nevertheless, once a satisfactory combination of columns is selected, and the final equation calculated, the substitutions and subsequent calculations can be done readily.

ACKNOWLEDGEMENT

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SUMMARY

It is shown that the method of "structural analysis" developed for paper chromatography by SCHAUER AND BULIRSCH gives analogous results using gas chromatography when the equations are corrected for gas chromatography parameters and the proper liquid phases are used for partitioning agents. Aliphatic amines were used to show the analogy.

REFERENCES

[1] H. K. SCHAUER AND R. BULIRSCH, *Z. Naturforsch.*, 13B (1958) 327.
[2] C. E. MELOAN AND R. W. KISER, *Problems and Experiments in Instrumental Analysis*, C. E. Merrill Company, Columbus, Ohio, 1963.
[3] C. MERRITT, JR., AND J. T. WALSH, *Anal. Chem.*, 34 (1962) 902.
[4] D. R. BEUERMAN AND C. E. MELOAN, *Anal. Chem.*, 34 (1962) 319.
[5] R. L. PECSOK, *Principles and Practice of Gas Chromatography*, Wiley, New York, 1959, pp. 137–8.

IDENTIFIZIERUNG ORGANISCHER SUBSTANZEN MIT HILFE DER GASCHROMATOGRAPHIE (CHROMATOGRAPHISCHE SPEKTREN)

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Wie bekannt wird die Gaschromatographie mit Erfolg zur Identifizierung organischer Verbindungen herangezogen. Als eine der ersten und häufig angewendeten Arbeitsmethoden ist die Vergleichung der Elutionszeiten zu nennen, die als Analogon zum Vergleichen der R_F-Werte bei der Papierchromatographie angesehen werden kann. Die Papierchromatographie hat im Vergleich mit der Gaschromatographie allerdings als weiteres Merkmal die charakteristische Färbung der Flecken für sich. Es ist bekannt, dass die Elutionszeiten auf einer stationären Phase unter gegebenen Versuchsbedingungen für eine Reihe von Verbindungen die gleichen sein können, so dass zum Vergleichen mit einer Standardverbindung (-substanz) ein weiteres Vergleichen mit noch einer anderen, womöglich mit mehreren stationären Phasen und den entsprechenden Elutionszeiten notwendig wird.

Die enge Beziehung des Verhaltens beim Chromatographieren zur chemischen Struktur und zum Charakter der stationären Phase wurde von einigen Autoren zur Identifizierung von Verbindungen durch Vergleichen der Elutionszeiten an zwei[1,2], gegebenenfalls an mehreren stationären Phasen benützt[3,4].

Aus den Unterschieden in den Elutionszeiten an den unterschiedlichen stationären Phasen kann auf die zu identifizierende Verbindung geschlossen werden. Diese Bestimmungen der einzelnen Elutionszeiten unter unterschiedlichen Bedingungen verlangen im Fall der Gaschromatographie ein zeitraubendes Auswechseln der Kolonnen und verbürgen kaum verlässlich reproduzierbare Versuchsbedingungen.

Seinerzeit wurde — um wieder auf die Papierchromatographie zurückzugreifen — eine papierchromatographische Methode der Identifizierung von nichtflüchtigen anorganischen und organischen Verbindungen aufgrund von sogenannten papierchromatographischen Spektren ausgearbeitet[5-7]. Hierbei wird zugleich unter reproduzierbaren Versuchsbedingungen in verschiedenen Lösungssystemen chromatographiert. Als Ergebnis der Analyse gibt dann die Verbindungslinie der korrigierten R_F-Werte — das "chromatographische Spektrum" — einen Kurvenverlauf, der charakteristisch ist für jede einzelne Verbindung. Diese Idee haben wir nun auf die Gas-Chromatographie übertragen.

Das Prinzip der Versuchsanordnung lehnt sich demgemäss an die eben erwähnte Arbeitsmethode der Papierchromatographie an: die Analysenprobe wird im Gaschromatographen gezwungen sich gleichmässig auf vier Kolonnen aufzuteilen, die stationäre Phasen unterschiedlicher Polarität besitzen. In diesen Kolonnen bewegt

sich nun die zu analysierende Probe je nach ihrer Struktur mit verschiedener Geschwindigkeit. Die Enden der einzelnen Kolonnen sind bei unserer Versuchsanordnung zu einem gemeinsamen Einlauf in einen Detektor vereinigt. Der Detektor zeichnet die Reihenfolge und den Verlauf der Elutionskurven aus den vier Kolonnen in üblicher Weise auf. Im Idealfall lässt sich so ein Chromatogramm mit vier Einzelkurven erhalten, die ein für jede Verbindung charakteristisches Maximum besitzen und im Zusammenhang mit der chemischen Struktur der Prüfsubstanz sind. Sind in zwei Kolonnen gleiche Elutionszeiten verzeichnet, dann enthält das Chromatogramm nur drei Kurven. Dieses Verhalten kann man, wie im Versuchsteil ausgeführt wird, zum Vergleichen des Chromatogramms mit einem Chromatogramm einer Standardsubstanz oder zum Einordnen der zu identifizierenden Verbindung in eine bestimmte Verbindungsklasse benützen.

BESCHREIBUNG DER VERSUCHE

(*a*) *Auswahl der stationären Phase*

Die stationäre Phase muss eine Reihe von Bedingungen erfüllen: Stabilität in jeder Hinsicht, Einsatzfähigkeit in einem grossen Temperaturintervall, im Vergleich zu den anderen benützten stationären Phasen hinreichende Unterschiedlichkeit in der Polarität angefangen von stark polarer Phase über zwei Phasen mittlerer Polarität bis zu unpolarer Phase.

Für jede der vier Kolonnen haben wir der Reihe nach vorläufig folgende stationäre Phasen als die besten befunden:

(1) 3,5-Dinitrobenzoylester des Trimethylenglykolbutyläthers (hergestellt im Forschungsinstitut für organische Synthesen, Pardubice-Rybitví).

(2) Polyäthylenglykoladipat (desgleichen).

(3) Silikon-Elastomer E 301 (Griffin & George, London).

(4) Destillationsrückstände von Methylphenylsilikonöl (Forschungsinstitut für organische Synthesen, Pardubice-Rybitví).

Diese vier Substanzen wurden einzeln in einer Menge von 20 Gewichtsprozent (bezogen auf das Gewicht des reinen Trägersubstanz) auf die Trägersubstanz aufgetragen.

(*b*) *Trägersubstanz*

Rysorb BLK (Hersteller Forschungsinstitut für organische Synthesen, Pardubice-Rybitví) getrocknet bei 130° in der Körnung 0.25–0.5 mm.

(*c*) *Apparatur*

Die Zusammenstellung ist aus Fig. 1 ersichtlich. Der Hauptteil der Apparatur ist die Gruppe der vier Kolonnen, die im Gaschromatograph mit üblichem Luftthermostat und beliebigem Detektor angeordnet sind. Die Fig. 1 entsprechend geht das Trägergas (Stickstoff) aus der Bombe A über den Manostat B, das Nadelventil C und das Manometer D in die Dosiervorrichtung. Diese ist im Prinzip ein kleiner Messingbecher mit Stopfen, wie es beim Dosieren in chromatographische Kolonnen üblich ist. Unter diesem Stopfen befindet sich ein kleines Messingsieb, das eine Verteilung der flüssigen Probesubstanz und Erleichtern des Verdampfens zur Aufgabe hat. Das Sieb verhindert ausserdem das Einspritzen grösserer Mengen von Substanz direkt in eine der Kapillaren F, deren Eingangsöffnungen in die Dosiervorrichtung münden. Die

Kapillaren F sind aus Metall, haben einen Innendurchmesser von 0.6 mm, gleiche Länge (11 cm) und gleichen dynamischen Widerstand, der grösser sein muss als der dynamische Widerstand der angeschlossenen, mit nach 1–4 imprägniertem (siehe oben) Rysorb BLK gefüllten Kolonnen G (Fig. 1). Diese vier Kolonnen sind aus

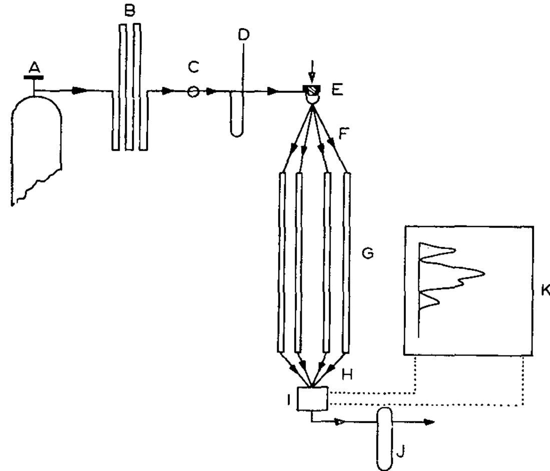

Fig. 1. Schematische Darstellung eines Apparates für chromatographische Spektren.

Glas, 90 cm lang, Innendurchmesser 5 mm. Ihre Enden sind wieder durch gleiche Kapillaren H mit dem gemeinsamen Detektor I verbunden. Der Strömungmesser J misst den Durchfluss des Trägergases, dessen Geschwindigkeit viermal grösser als bei Benützung einer einzelnen Kolonne gewählt wird. Bei unseren Versuchen war die Gasgeschwindigkeit 200 ml/min. Das Schreibgerät K verzeichnet wie üblich die Abhängigkeit der Konzentration der analysierten Substanz im Detektor nach der Zeit.

AUSFÜHRUNG DER IDENTIFIZIERUNG EINER UNBEKANNTEN VERBINDUNG

Für die gleichzeitige Elution an vier Kolonnen wählten wir einige Arbeitstemperaturen des Thermostaten entsprechend den Siedepunkten der zu untersuchenden Stoffe. Diese sind 55°, 85°, 110° und 150°, sodass damit ein Bereich bis zum Siedepunkt 200° der zu untersuchenden Verbindung umfasst werden kann. Die Prüfsubstanz, die zum Zweck der Identifizierung wenigstens in 90 %-iger Reinheit vorliegen soll, wird in üblicher Weise mit einer Injektionsspritze in die Dosiervorrichtung E eingespritzt.

Die Menge der Prüfsubstanz wird entsprechend der Anzahl der eingesetzten Kolonnen und der Art der Detektion bemessen. Zur Kontrolle der Apparatur wird von Zeit zu Zeit Benzol als Standardsubstanz gemessen und die Elutionsdaten der zu identifizierenden Verbindungen werden sodann an Hand dieser Eichungen korrigiert. Alle gemessenen Elutionsvolumina sind um das tote Volumen der Apparatur zu korrigieren; das tote Volumen der Apparatur wird aus der Elutionszeit von Wasserstoff bestimmt.

Die bei unseren Versuchen gemessenen Elutionszeiten sind in den Diagrammen in Fig. 2–5 dargestellt. Die Fig. 6–8 zeigen die chromatographischen Spektren verschiedener Stoffe.

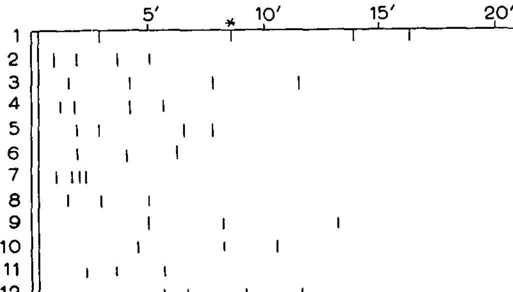

Fig. 2. Schematische Darstellung der chromatographischen Spektren bei 55°. 1 = Benzol; 2 = *n*-Hexan; 3 = 2,2,4-Trimethylpentan; 4 = Methanol; 5 = Äthanol; 6 = Aceton; 7 = Diäthylether; 8 = Diisopropyläther; 9 = Chloroform; 10 = Tetrachlorkohlenstoff; 11 = Äthylformiat; 12 = Äthylacetat.

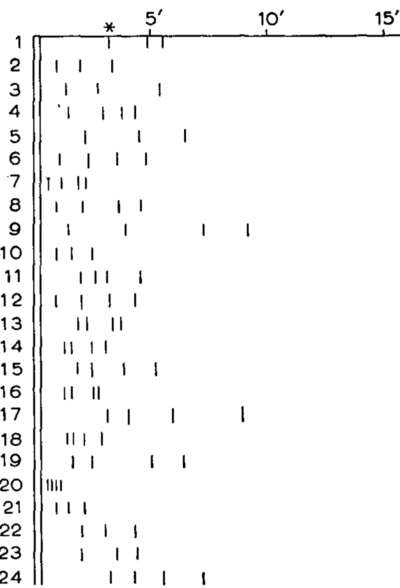

Fig. 3. Schematische Darstellung der chromatographischen Spektren bei 85°. 1 = Benzol; 2 = Cyclohexan; 3 = Methylcyclohexan; 4 = Cyclohexen; 5 = Methylcyclohexen; 6 = 2,2,4-Trimethylpentan; 7 = *n*-Hexan; 8 = *n*-Heptan; 9 = *n*-Oktan; 10 = Aceton; 11 = Methylethylketon; 12 = Diacetyl; 13 = Äthylacetat; 14 = Äthanol; 15 = *n*-Propylalkohol; 16 = Isopropylalkohol; 17 = Isobutylalkohol; 18 = *tert.*-Butanol; 19 = Allylalkohol; 20 = Diäthyläther; 21 = Diisopropyläther; 22 = Chloroform; 23 = Tetrachlorkohlenstoff; 24 = Thiofen.

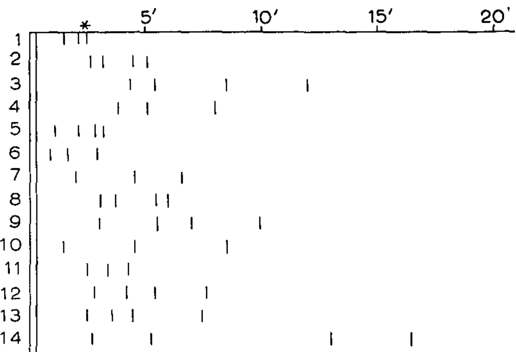

Fig. 4. Schematische Darstellung der chromatographischen Spektren bei 110°. 1 = Benzol; 2 = Toluol; 3 = *m*-Xylol; 4 = Äthylbenzol; 5 = Methylcyclohexen; 6 = Methylcyclohexan; 7 = Dibutyläther; 8 = Butylacetat; 9 = Methylchloracetat; 10 = Äthylchloracetat; 11 = Methylisobutylketon; 12 = Amylalkohol; 13 = Isoamylalkohol; 14 = Pyrrol; 15 = Dioxan.

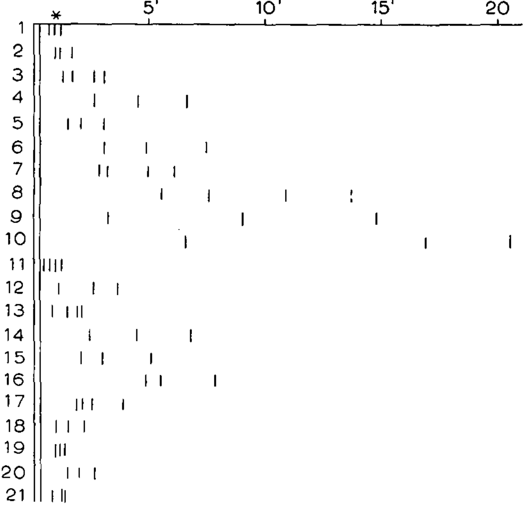

Fig. 5. Schematische Darstellung der chromatographischen Spektren bei 150°. 1 = Benzol; 2 = Toluol; 3 = *p*-Xylol; 4 = *p*-Cymol; 5 = Äthylbenzol; 6 = Butylbenzol; 7 = Brombenzol; 8 = Jodbenzol; 9 = Benzonitril; 10 = Nitrobenzen; 11 = Nitromethan; 12 = Isoamyläther; 13 = Dibutyläther; 14 = Cyclohexanol; 15 = Hexylalkohol; 16 = 2-Äthylhexanol; 17 = 2-Äthylbutanol; 18 = Isoamylalkohol; 19 = Methyläthylketon; 20 = Amylacetat; 21 = Thiofen.

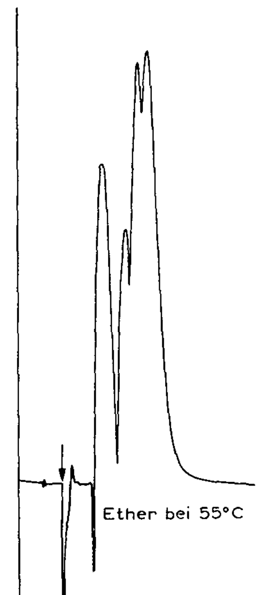

Fig. 6. Chromatographisches Spektrum von Äther bei 55°.

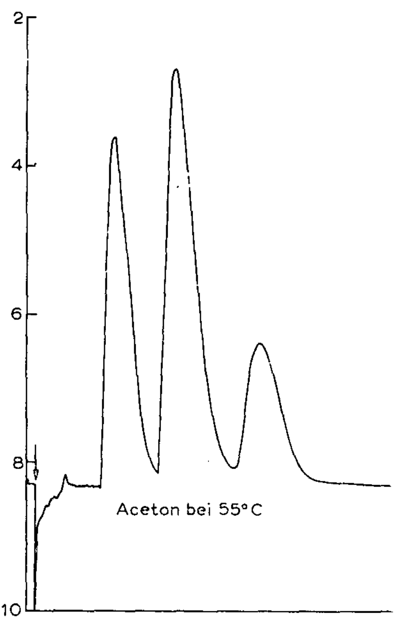

Fig. 7. Chromatographisches Spektrum von Aceton bei 55°.

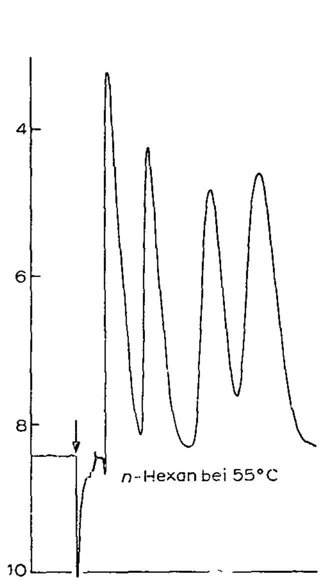

Fig. 8. Chromatographisches Spektrum von *n*-Hexan bei 55°.

DISKUSSION DER ERGEBNISSE

Wie ersichtlich, ist das Ergebnis dieser Arbeitsweise ein Bündel von drei bis vier chromatographischen Kurven mit Maxima, die in verschiedenen Abständen auftreten. Wir haben diese Diagramme in Analogie zur Papierchromatographie vorläufig mit dem Ausdruck "chromatographische Spektren" bezeichnet obwohl diese Bezeichnung nicht sehr glücklich ist[6] und der Ausdruck "chromatographisches Profil" unseres Erachtens besser entsprechen würde.

Aus den Diagrammen in Fig. 2–5 ist ersichtlich, dass praktisch genommen (mit Ausnahme einiger isomerer Verbindungen) keine einzige der untersuchten Verbindungen ein chromatographisches Spektrum aufweist, das identisch wäre mit dem Spektrum einer anderen unter den untersuchten Verbindungen. Wenn auch die Elutionszeiten bei einzelnen Kolonnen die gleichen sind, sind sie wieder bei anderen Kolonnen verschieden, so dass die Unterschiedlichkeit des Gesamtergebnisses jeder Messung gegenüber der einer anderen Verbindung bestehen bleibt. Haben wir also eine Standardsubstanz zur Verfügung, so sind wir in der Lage rasch und sicher einen Vergleich der Spektren sowohl der Standardsubstanz als auch der Prüfsubstanz vorzunehmen und so die Prüfsubstanz zu identifizieren. Selbstverständlich kann man auch das chromatographisches Spektrum der Prüfsubstanz mit ebensolchen Spektren aus der Sammlung am Arbeitsplatz vergleichen.

Wie schon vorhin erwähnt existiert eine enge Beziehung zwischen der chemischen Struktur der untersuchten Verbindung, den Eigenschaften der stationären Phase und der Elutionszeit. Diese Tatsache lässt sich zur Identifizierung von Substanzen und Verbindungen verwerten, von denen kein Vergleichsstandard zur Verfügung steht.

Zu diesem Zweck wird eine Eichung einer Kolonne und einer stationären Phase ein für allemal mit Benzol als Testsubstanz vorgenommen (die zugehörige Elutionszeit ist in den Fig. 2–5 mit einem Stern bezeichnet). Auf Basis der Elutionszeit des Benzols lassen sich sodann die relativen Elutionszeiten für beliebige Substanzen und Temperaturen berechnen.

Die Differenzen der Logarithmen dieser Elutionszeiten (z.B. zwischen dem ersten und zweiten, oder dem zweitem und dritten, oder dem ersten und vierten Maximum) sind, wie das Diagramm zeigt, bei ähnlichen Stoffen praktisch sehr ähnlich. Wenn wir also die Elutionszeiten der zweiten und ersten Maxima mit r_2 und r_1 bezeichnen, so gilt:

$$\log r_2 - \log r_1 = \Delta R$$

Das Ergebnis der Berechnungen von ΔR für eine Reihe von relativen Elutionszeiten ist die Tabelle I, und das Diagramm in Fig. 9. Durch Berechnung von ΔR für das erste und zweite, zweite und dritte, erste und letzte Maximum kann man eine unbekannte Substanz in eine der im Diagramm in Fig. 9 verzeichneten Gruppen einreihen. Es darf nicht verschwiegen werden, dass in einigen wenigen Fällen das Ergebnis nicht eindeutig ist und dass zwei Gruppen für die betreffende Substanz als Analysenergebnis in Betracht kommen. Solche Fälle sind allerdings selten. Aus dem Diagramm in Fig. 9 ist noch die Häufigkeit des Auftretens der einzelnen Werte zu entnehmen, die sich durch weitere Angaben noch präzisieren lassen.

Mit Hilfe der hier angegebenen Methode kann man also eine unbekannte Substanz

TABELLE I

Substanz	*I–II Maximum*				*II–III Maxmium*				*I–letztes Maximum*			
	55°	*85°*	*110°*	*150°*	*55°*	*85°*	*110°*	*150°*	*55°*	*85°*	*110°*	*150°*
Benzol	26.0	10.0	10.0	9.0	11.0	3.0	3.0	7.0	41.0	10.0	13.0	16.0
Toluol			4.5	6.0			9.0	6.5			16.5	17.5
m-Xylol			7.0				10.5				23.0	
p-Xylol				6.0				11.0				17.0
Äthylbenzol			7.0	6.0			9.5	10.0			17.5	16.0
p-Cymol				13.0				8.5				20.5
Butylbenzol				12.0				9.5				21.0
2,2,4-Trimethylpentan	21.0	24.0			13.5	10.5			45.0	42.0		
n-Hexan	16.0	18.0			16.0	8.5			39.0	35.0		
n-Heptan		20.0				12.0				38.0		
n-Oktan		23.5				14.0				43.0		
Cyclohexan		21.0				13.0				34.0		
Methylcyclohexan		18.0	13.0			16.0	27.0			33.5	29.0	
Cyclohexen		18.0				6.0				27.0		
Methylcyclohexen		18.0	16.0		7.5	7.0				25.5	25.5	
Aceton	18.0	12.5			10.5	12.0			28.5	24.5		
Methyläthylketon	8.5			5.5		3.0		4.5		20.0		10.0
Methylisobutylketon			9.0				5.0				14.0	
Methylalkohol	9.5				21.0				38.0			
Äthylalkohol	10.0	8.0			19.5	15.5			34.0	26.5		
n-Propylalkohol		7.0				10.0				24.5		
Isopropylalkohol		6.5				12.0				20.5		
Isobutylalkohol		6.0				9.5				24.0		
tert.-Butylalkohol		5.0				6.0				17.5		
Allylalkohol		9.0				17.5				32.0		
Amylalkohol			7.0				6.0				21.0	
Isoamylalkohol			9.0	13.0			5.0	10.0			25.5	24.0
Cyclohexanol				13.5				19.5				33.0
Hexylalkohol				8.5				13.0				21.0

Diäthyläther	14.0	1[illegible].0			4.0	5.5			21.0	18.0		
Diisopropyläther	15.5	16.0			12.5	8.5			28.0	24.0		
Dibutyläther			20.5	20.0			8.5	7.0			29.0	29.0
Isoamyläther				24.0				8.0				32.0
Chloroform	11.5	10.5			10.5	8.5			21.5	18.0		
Tetrachlorkohlenstoff	14.0	13.0			5.5	6.5			19.5	18.5		
Brombenzol				3.0				10.5				18.5
Jodbenzol				7.0				9.0				20.0
Äthylformiat	10.0				11.0				20.5			
Äthylacetat	3.0	4.0			7.0	9.0			16.5	16.5		
Butylacetat			5.0				8.5				16.0	
Amylacetat				9.5				9.0				18.0
Thiophen		7.0		9.0		6.0		3.0		19.0		12.5
Pyrrol			17.0				21.0				42.5	
Diacetyl		20.5				12.0				39.5		
Dioxan			12.5				8.0				20.5	
Nitromethan				30.0				10.0				66.0
Nitrobenzol				13.0				15.0				27.5
Benzonitril				23.5				12.0				35.0
Chlormethylacetat			14.5								27.5	
Chloräthylacetat			27.5								47.5	

schnell mit einem bekannten Standard vergleichen, oder wenn keine Standardsubstanz zur Verfügung steht, kann man wenigstens eine Zuordnung der Prüfsubstanz in bekannte Verbindungsgruppen vornehmen. Wenn man die Elutionszeit jeder einzelnen der vier Kolonnen der Apparatur besonders bestimmten könnte,

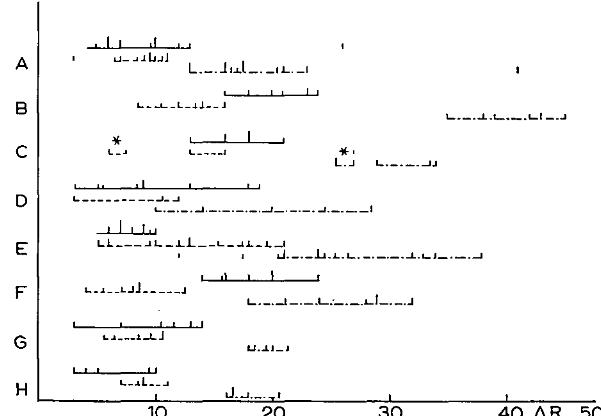

Fig. 9. Graphische Darstellung von ΔR-Werten für verschiedene Gruppen der Verbindungen. A = Aromatische Kohlenwasserstoffe; B = Aliphatische Kohlenwasserstoffe; C = Cyclische Kohlenwasserstoffe; D = Ketone; E = Alkohole; F = Äther; G = Halogenkohlenwasserstoffe; H = Ester. ——— ΔR zwischen dem I–II Maximum; - - - - - ΔR zwischen dem II–III Maximum; —·—·—· ΔR zwischen dem I– letzten Maximum.

wäre von jeder der untersuchten Substanzen viel mehr auszusagen, aber damit ginge ein grosser Vorteil der hier beschriebenen Methode verloren: die Möglichkeit der Anwendung der hier beschriebenen Methode an Gaschromatographen beliebiger Konstruktion, Herkunft und Detektion, und das ohne nennenswerte Änderungen an der Apparatur.

Die Anzahl der gleichzeitig eingesetzten Kolonnen kann beliebig gross sein, wir glauben aber, dass vier Kolonnen für die üblichen Ansprüche ausreichend sind. Selbstverständlich lassen sich auch andere stationäre Phasen als die hier angegebenen verwenden; es ist aber zu bedenken, dass Vergleichen von Analysenergebnissen verschiedener Arbeitsplätze miteinander und ihre vollkommene Auswertung gleiche Arbeitsbedingungen zur Voraussetzung haben, d.h. also gleiche Trägersubstanzen, stationäre Phasen und gleiche Länge der Kolonnen.

Die hier beschriebene Methode ist nicht als selbständig anzuwendende Methode der Identifikation unbekannter Verbindungen gedacht, sondern als bequem auszuführende Ergänzung bekannter Bestimmungsmethoden.

ZUSAMMENFASSUNG

Es wird eine Methode zur Identifizierung flüchtiger organischer Substanzen mit Hilfe der Gaschromatographie ausgearbeitet, die auf der gleichzeitigen Elution der Substanz aus vier mit verschiedenen stationären Phasen gefüllten Kolonnen beruht. Das auf diese Weise erhaltene "chromatographische Spektrum" ist charakteristisch für eine jede Substanz und kann zur Identifizierung unbekannter Substanzen dienen.

Die Identifizierung kann durch Vergleichen mit "Spektren" bekannter Substanzen vor sich gehen, oder es kann die Lage der Maxima der Elutionskurven zur Auffindung von Verbindungsgruppen dienen, zu denen die unbekannte Substanz gehört.

SUMMARY

A method has been developed for the identification of volatile organic compounds by means of gas chromatography. This method is based on eluting the compound simultaneously from four columns filled with different stationary phases. The "chromatographic spectrum" obtained in this way is characteristic for each compound and can serve to identify unknown compounds. Identification can be carried out by comparing the "spectrum" with those of known substances, or the positions of the maxima of the elution curves can be used to determine the class of compounds to which the unknown belongs.

LITERATUR

[1] A. T. JAMES, A. J. P. MARTIN UND G. SMITH, *Biochem. J.*, 52 (1952) 238.
[2] G. J. PIEROTTI, C. H. DEAL, E. L. DERR UND P. E. PORTER, *J. Am. Chem. Soc.*, 78 (1956) 2989.
[3] R. A. HIVELY, *J. Chem. Eng. Data*, 5 (1960) 237.
[4] S. M. CSICSERY UND H. PINES, *J. Chromatog.*, 9 (1962) 34.
[5] J. FRANC UND Z. STRÁNSKÝ, *Collection Czech. Chem. Commun.*, 24 (1959) 3611.
[6] J. FRANC, in *Some General Problems of Paper Chromatography*, ČSAV, Prag, 1962, S. 189.
[7] M. LEDERER, in *Some General Problems of Paper Chromatography*, ČSAV, Prag, 1962, S. 181.

A SIMPLE, GENERAL METHOD FOR GRADIENT ELUTION USING ELUENTS OF UNEQUAL DENSITY

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INTRODUCTION

Generally when complex mixtures of compounds have to be fractionated by column chromatography it is advantageous to elute with a continuous concentration gradient[1]. This makes the fractionation automatic and easily reproducible, and avoids the formation of spurious peaks that occurs when discontinuous gradients are used. It reduces tailing and improves resolution, moreover, if the type of gradient used is well chosen. (Gradients may be classified[2,3] as linear, convex, concave, or compound.) Despite these advantages continuous gradient elution is not yet widely used for natural lipid mixtures. One reason why this is so appears to be a misconception, based upon reports about convex gradients, that all continuous gradients inevitably resolve lipids less well than discontinuous gradients[2]. Another reason is the paucity of apparatus producing linear and concave gradients with eluents of unequal density[4].

In the method of LAKSHMANAN AND LIEBERMAN[5,6] the column is fed from a stirred reservoir which initially contains the less polar eluent, and to which the more polar eluent is added at a controlled rate; the addition may be made by means of a motor-driven syringe[7]. Linear[8] and concave[9] gradients may also be produced by means of metering pumps. These methods all require a constancy of flow rate through the column that is often difficult to achieve.

Apparatus of the type first described by PARR[10] is probably the simplest and most versatile available for producing continuous gradients. It consists (Fig. 1) of

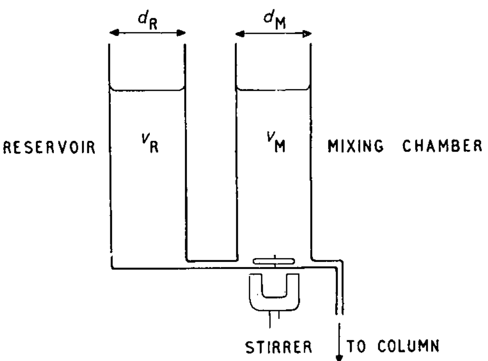

Fig. 1. Parr's apparatus[10].

two vertical, parallel-walled vessels, open to the atmosphere and joined below by a narrow tube. With efficient stirring and slow delivery the system may be considered always at hydrostatic equilibrium. Then the concentration, c, of the more polar eluent in the mixing chamber (and being delivered to the column) when a volume v has been delivered is given by the equation:

$$c = c_R - (c_R - c_M) \cdot \left(1 - \frac{v}{v_R + v_M}\right)^{v_R/v_M} \quad (1)$$

where v_R and v_M are the volumes initially present in the reservoir and mixing chamber respectively, and c_R and c_M the initial concentrations of the more powerful eluent. Equation (1) is known[11-13] to apply to liquids of equal density that do not change in volume when mixed. Since $v_R/v_M = d_R^2/d_M^2$ linear gradients are predicted when $d_R = d_M$ (internal diameter of reservoir = internal diameter of mixing chamber), convex when $d_R > d_M$, and concave when $d_R < d_M$. Convexity and concavity are slightly greater after the half-way marks [$v = (v_R + v_M)/2$] than before[3,11,12].

The above predictions fail for liquids of unequal density. For example, if $d_R = d_M$ one volume of chloroform in the mixing chamber requires nearly two volumes of methanol in the reservoir to establish hydrostatic equilibrium: then a convex, not a linear gradient is produced. The hydrostatic problem can be solved[14,15] by using motor-driven syringes in place of open vessels, but no such apparatus has been described that is suitable for organic solvents. (Large PTFE pistons[16] would be necessary.)

METHOD

It has now been found that when eluents of unequal density are used in apparatus of the type shown in Fig. 1 the gradients produced are described well by an empirically modified equation,

$$c = c_R - (c_R - c_M) \cdot \left(1 - \frac{v}{v_R + v_M}\right)^{\rho_M d_R^2/\rho_R d_M^2} \quad (2)$$

where ρ_R is the density of the liquid in the reservoir and ρ_M that of the liquid initially present in the mixing chamber. The gradients are:

linear when $\rho_M d_R^2 = \rho_R d_M^2$,
convex when $\rho_M d_R^2 > \rho_R d_M^2$,
and *concave* when $\rho_M d_R^2 < \rho_R d_M^2$.

Gradients can be designed and prepared quite simply in the following way.

(i) Choose the initial and final concentrations (c_M, c_R) of the more powerful eluent.

(ii) Decide upon convexity, concavity, or linearity by choosing the concentration (c_H) at the half-way mark.

(iii) Calculate the ratio of the diameters from the derived equation:

$$\frac{d_R}{d_M} = \sqrt{\frac{\rho_R}{\rho_M} \cdot \frac{-1}{\log 2} \cdot \log\left(\frac{c_R - c_H}{c_R - c_M}\right)} \quad (3)$$

This is done by means of a nomogram (Fig. 2).

(iv) Choose approximate volumes (v_R, v_M).

(v) Select two reasonably cylindrical vessels that will contain these volumes and fulfil the diameter ratio.

(vi) Connect the vessels and proceed as indicated below.

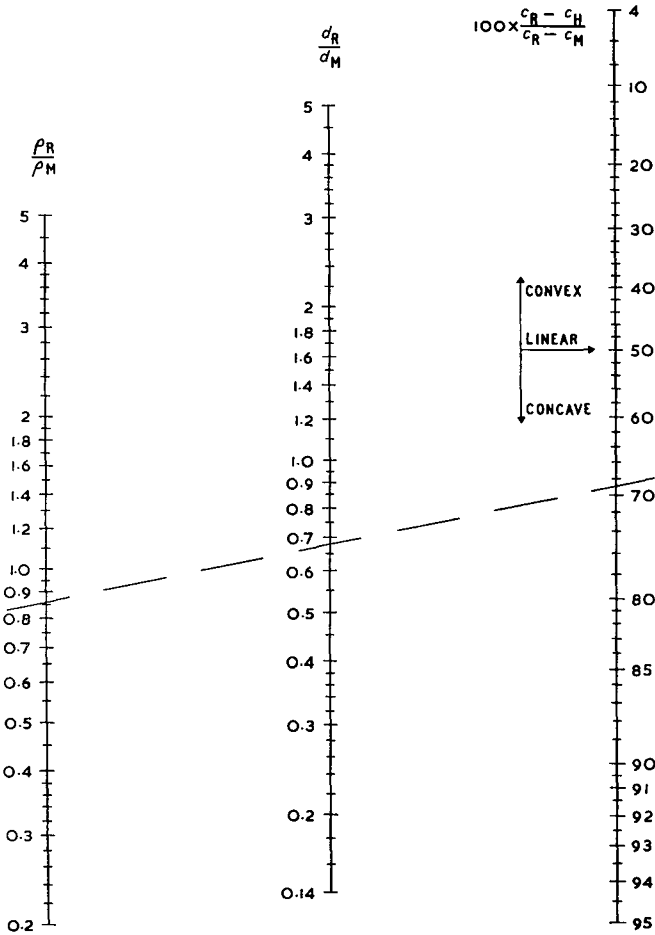

Fig. 2. Nomogram for eqn. (3). The dashed line illustrates the computation of the diameter ratio for a concave gradient, 0–30 % methanol in chloroform, $c_H \doteq 10\%$.

A typical arrangement for a concave gradient of methanol in chloroform is shown in Fig. 3. The reservoir is a 2 l measuring cylinder and the mixing chamber a Winchester bottle. Connecting the mixing chamber to the reservoir and to the column are siphon tubes (K, L), which are made of capillary glass (I.D. 2 mm) and have looped side pieces (S, T) closed with PTFE-barrelled stopcocks or screw clips on plastic sleeves.

The *starting procedure* is as follows.

(i) Place the liquids in R and M, including a 10 % excess over v_R in R to allow for minor errors in measurement and computation.

(ii) Close S and T.

(iii) Start the siphon through L, by applying a vacuum line to the lower end, and connect it to the column.

(iv) Start the stirrer.

(v) Start the siphon through K by connecting the vacuum line to S and opening the stopcock briefly.

(vi) Raise R to cause visible flow into M, and then lower it to the minimum height necessary for flow to continue.

(vii) Close R and M with tight cotton-wool plugs and aluminium foil.

(viii) Remove any air bubbles that rise in L from the column by applying the vacuum line through T.

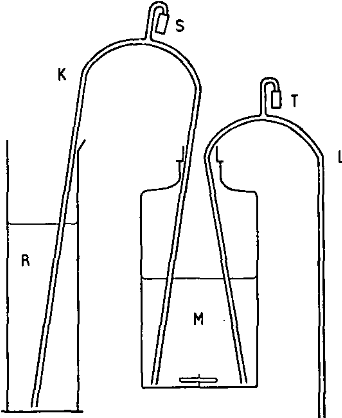

Fig. 3. Apparatus producing a continuous, concave gradient of methanol in chloroform.

The arrangement shown in Fig. 3 is suitable whenever the less polar eluent is the denser. But when it is the lighter an upward-screw mechanical stirrer or (preferably) an upward impelling vibratory mixer should be used. The lighter liquid in M may render the siphon useless by rising in currents in K, despite the opposing flow from R. This difficulty can be overcome by directing the outlet from K upwards: some alternative arrangements for connecting the vessels are shown in Fig. 4. The tubes must always be arranged so that L cannot take in unmixed liquid.

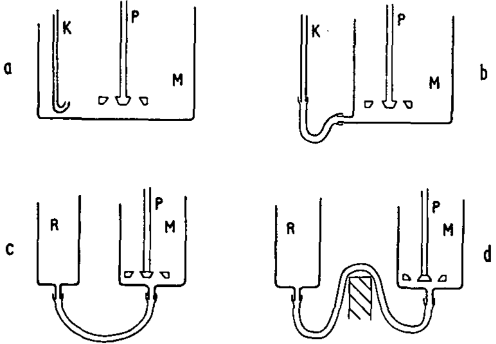

Fig. 4. Some alternative connecting arrangements for the apparatus illustrated in Fig. 3: (a), (b), and (c) are suitable when the more polar eluent is the denser, and (d) when it is the less dense. Flexible connections are made with narrow PTFE tubing. P is a vibratory mixer.

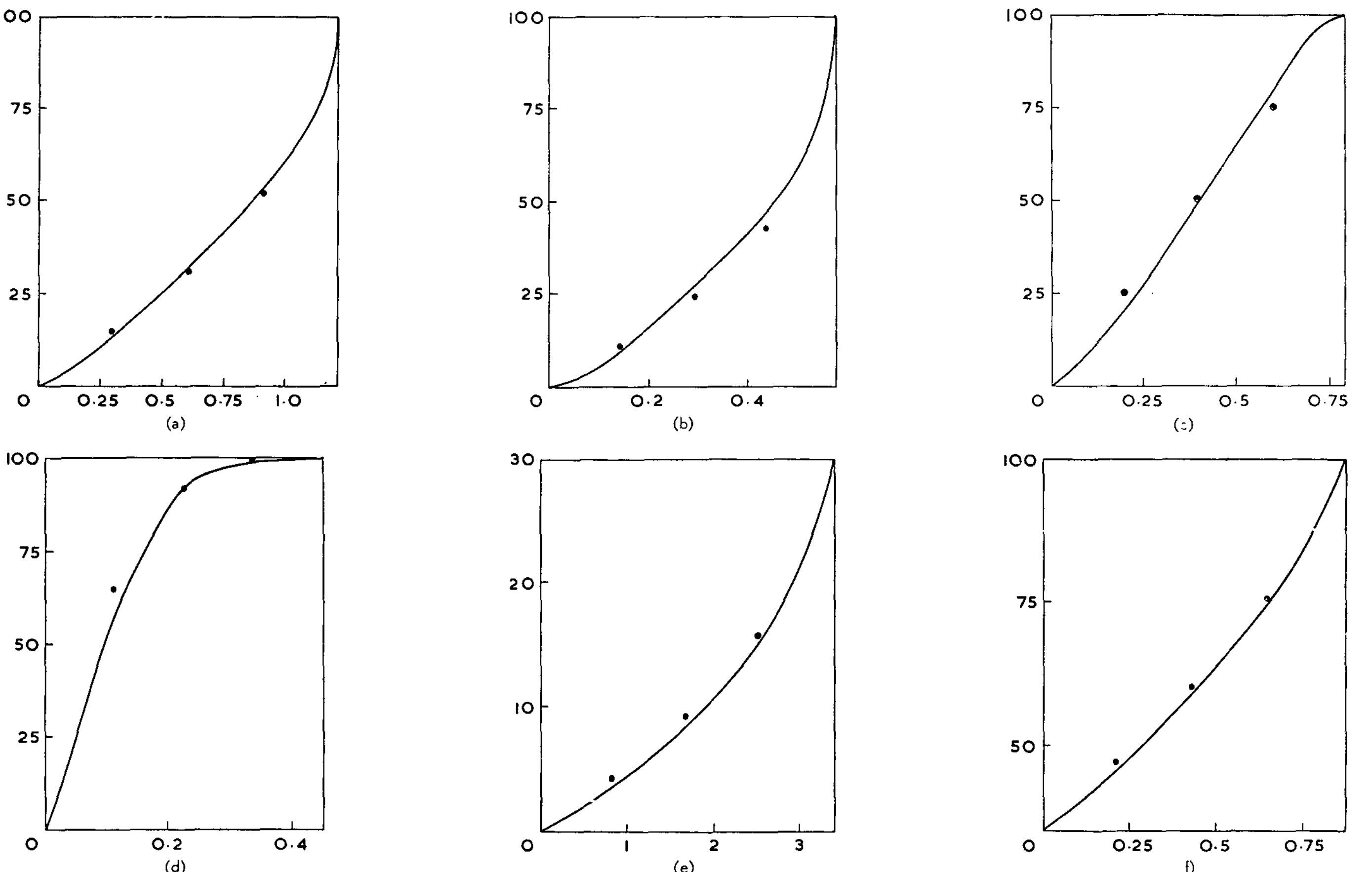

Fig. 5. Gradients produced by the method described. Ordinates: % (v/v) of more powerful eluent. Abscissae: volume (l) delivered to column. Points (●) calculated from d_R, d_M, ρ_R, ρ_M. Gradient (b) was determined ($D_{600\ m\mu}$) as Sudan Black, which was dissolved (10 μg/ml) in the ethanol; the remainder were determined by refractive index measurements. (a) Concave, 0–100 % diethyl ether in petroleum ether. Reservoir: 16 oz. reagent bottle (d_R = 75 mm). Mixing chamber: Mallinckrodt silicic acid bottle (d_M = 100 mm). (b) Concave, 0–100 % ethanol in diethyl ether. Reservoir: wash bottle (d_R = 50 mm). Mixing chamber: reagent bottle (d_M = 75 mm). (c) Linear, 0–100 % chloroform in petroleum ether. Reservoir: Winchester bottle (d_R = 121 mm). Mixing chamber: reagent bottle (d_M = 80 mm). (d) Convex, 0–100 % chloroform in petroleum ether. Reservoir: 1 l bottle (d_R = 96 mm). Mixing chamber: chromatographic column tube (d_M = 34 mm). (e) Concave, 0–30 % methanol in chloroform. (Diameter ratio computed in Fig. 2 and apparatus illustrated in Fig. 3.) Reservoir: 2 l measuring cylinder (d_R = 79 mm). Mixing chamber: Winchester bottle (d_M = 116 mm). (f) Concave, 35–100 % methanol in chloroform. Reservoir: wash bottle (d_R = 64 mm). Mixing chamber: 1 l bottle (d_M = 96 mm).

RESULTS AND DISCUSSION

A selection of gradients that have been produced by this method and measured is shown in Fig. 5. Clearly the method is applicable for linear, convex, and concave gradients, and whether the more or the less polar eluent is the denser. Since the basis of the calculations is empirical and since the glassware used did not have strictly regular geometry, the closeness of the calculated points to the observed curves in Fig. 5 is gratifying; and for most column chromatographic work it is fully adequate. For quantitative work with micro columns the use of tubes of accurately measured dimensions, instead of bottles etc., would probably be justified.

It is interesting that BILLIMORIA *et al.*[17] have already proved that a linear gradient of methanol in chloroform was produced when the condition $\rho_M d_R^2 = \rho_R d_M^2$ was satisfied. BADER AND MORGAN[18] recently found that equation (2) predicted very well a concave gradient of methanol in chloroform. However, they did not apparently realize its more general implications. KOČENT[19] has already proposed nomograms for gradient elution but their application to eluents of unequal density would be very laborious. His equation differs from equation (2) and predicts less accurately, seemingly[20] because he neglected a density term in the development.

The concave gradients shown in Fig. 5 are all more concave after the half-way mark than before. This situation can be reversed if the cylindrical reservoir is replaced by an upright conical flask. Such gradients, like that shown in Fig. 6 (see also ref. 4),

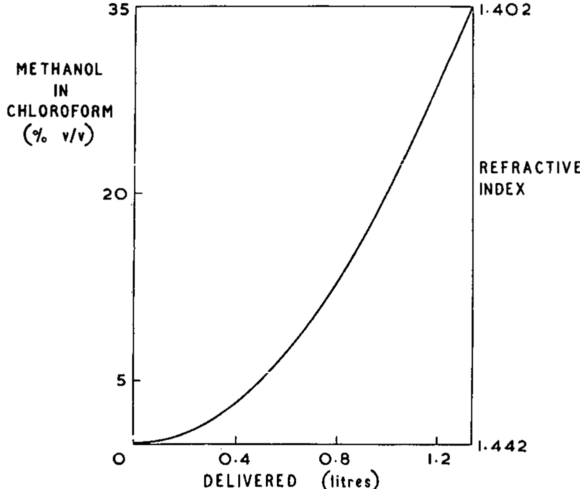

Fig. 6. Concave gradient, 0–35 % methanol in chloroform. Reservoir: 0.5 l *conical* flask (maximum d_R = 95 mm). Mixing chamber: 1 l cylindrical bottle (d_M = 96 mm).

are needed when the mixture to be chromatographed contains a preponderance of compounds that are eluted by low concentrations of the more polar eluent[21]. In other circumstances upright or inverted conical flasks may find use as reservoirs or as mixing chambers.

Gradient elution as advocated in this paper has fully proved its value for silicic acid chromatography of washed total lipid extracts, although stepwise elution is

still often preferable[21] for rechromatography of isolated fractions. It must be emphasized that during gradient elution the concentration of the more powerful eluent in the effluent at any instant is often very much less than that in the eluent entering the top of a column. With certain reservations, the ultimate factor determining whether or not a compound is eluted is the concentration in the effluent. Thus the gradient shown in Fig. 6, which ended at 35 % methanol, did not elute lecithins from a column containing 45 g of silicic acid although these compounds can be eluted with 20 % methanol[2].

In adsorption chromatography the concentration in the effluent normally lags behind that in the eluent by more than the column volume because, in order to maintain adsorption equilibrium, the solid phase is continually attaching more of the more polar eluent. *Continuous* behaviour of this kind is unobjectionable, and may indeed be vital to separation processes. But if before gradient elution is begun the solid phase contains none of the more polar eluent, none will appear in the effluent until the whole column has reached equilibrium. (Adsorbents such as silicic acid and alumina are well known for their ability to remove polar solutes from non-polar solvents until they become saturated.) Then there will be a *discontinuous*, steep rise in concentration, very likely causing the elution of several compounds in a single, sharp peak. It is therefore a good practice to prepare columns using a 50:50 mixture of the eluents and wash them with 1 column volume of the less polar eluent before use.

SUMMARY

Problems of applying continuous gradient elution to the adsorption chromatography of lipids and other lipophilic compounds are discussed. A simple and versatile method for producing continuous concentration gradients from pairs of eluents of unequal density is described. The method makes use of a nomogram and common pieces of laboratory glassware, such as bottles, measuring cylinders, and chromatographic column tubes. It has been found satisfactory for linear, convex, and concave gradients whichever eluent is the denser.

REFERENCES

1 E. Heftmann, *Chromatography*, Reinhold, New York, 1961.
2 J. J. Wren, *J. Chromatog.*, 4 (1960) 173; *Chromatographic Reviews*, Vol. 3, Elsevier, Amsterdam, 1961, pp. 111, 177.
3 O. Mikeš, *Chem. Listy*, 54 (1960) 576.
4 J. J. Wren, *Nature*, 184 (1959) 816.
5 T. K. Lakshmanan and S. Lieberman, *Arch. Biochem. Biophys.*, 53 (1954) 258.
6 H. K. Mitchell, M. Gordon and F. A. Haskins, *J. Biol. Chem.*, 180 (1949) 1071.
7 P. Vestergaard, *J. Chromatog.*, 3 (1960) 560.
8 D. F. H. Wallach, J. Soderberg and L. Bricker, *Cancer Res.*, 20 (1960) 397.
9 G. J. Nelson, *J. Lipid Res.*, 3 (1962) 256.
10 C. W. Parr, *Biochem. J.*, 56 (1954) xxvii.
11 R. M. Bock and N.-S. Ling, *Anal. Chem.*, 26 (1954) 1543.
12 B. Drake, *Arkiv Kemi*, 8 (1955) 1.
13 P. Lebreton, *Bull. Soc. Chim. France*, (1960) 2188.
14 R. M. Bock, quoted by C. De Duve, J. Berthet and H. Beaufay, *Progr. Biophys. Biophys. Chem.*, 9 (1959) 325.
15 G. L. Choules, *Anal. Biochem.*, 3 (1962) 236.
16 A. C. Arcus, *J. Chromatog.*, 3 (1960) 411.
17 J. D. Billimoria, R. G. Curtis and N. F. Maclagan, *Biochem. J.*, 78 (1961) 185.
18 H. Bader and H. E. Morgan, *Biochim. Biophys. Acta*, 57 (1962) 562.
19 A. Kočent, *J. Chromatog.*, 6 (1961) 324.
20 G. White and J. J. Wren, unpublished.
21 J. J. Wren, D. S. Holub, A. D. Szczepanowska and W. Stern, in preparation.

DETERMINATION OF ERRORS IN A DENSITOMETRIC ANALYSIS OF GLYCINE ON PAPER CHROMATOGRAMS

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In an investigation on the sequence of amino acids in peptides, it became necessary to make quantitative analyses on the hydrolyzates of simple peptides. The primary object of this investigation was to distinguish between dinitrophenyl-glycyl-glycine (DNP-gly-gly) and DNP-gly-gly-gly. Taking DNP-gly as unity, it was only necessary to obtain the number of glycine units bound to it with a precision of $\pm$ 20 %.

In the method to be described a recording densitometer without filters was used. On account of this circumstance, it was expected that the errors would be large and experiments were carried out to determine their magnitude. However if a densitometer is used in combination with an electronic area integrator, the method is probably the most rapid quantitative analysis of amino acids on paper chromatograms available. In the present work, only the maximum error under fixed experimental conditions, using manual area measurements, has been established. The method therefore provides a practical means of establishing the sequence of amino acids in peptides when only very small quantities are available, and where the maximum error under fixed experimental conditions would be within the limits necessary to give an unambiguous answer.

Direct photometric evaluation of spots on paper chromatograms has been made by numerous investigators[1–34] in the past. According to INGLE AND MINSHALL[24], the most precise method of evaluation is by reflectance on dry paper. These authors also recommend transmittance through dry paper, while they discard both reflectance on and transmittance through paper made transparent by impregnation. Although maximum spot density methods[1,3,6,31] involve much less work, their limitations have been discussed by several authors[3,15,27].

The procedures employing maximum spot density times area[1,6,27] have some advantages, but are in general less accurate than the procedure of total scanning and curve integration, which is the method used here. However, the latter involves a considerable amount of manual work if no electronic integrator is available[38].

EXPERIMENTAL

Apparatus

An electronic densitometer, Model 525 of Photovolt Corporation*, consisting of:

(1) a transmission density unit, Model 52-C, for chromatography;

(2) a photometer, Model 501-A; and

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* Photovolt Corporation, 95 Madison Ave., New York 16, N.Y., U.S.A.

(3) a Varicord variable response recorder, Model 42-A. This recorder is equipped with a variable response selector switch with twelve positions. In position 1 it works as a conventional millivolt recorder. All other positions introduce curvature, with position 6 closely approximating the logarithm of the input.

Application and development of spots

Three solutions of chromatographically pure glycine were prepared: 0.10 mg/ml; 0.50 mg/ml and 1.00 mg/ml. For the first two solutions, 0.5 to 1.5 mg of glycine were weighed out to the nearest 0.02 mg, while for the most concentrated solution 5 mg was weighed out in the same way. Each sample was dissolved in an appropriate amount of 30 % isopropyl alcohol in distilled water to yield solutions of the above concentrations. The estimated errors for these solutions are:

0.10 mg/ml	3.7 %
0.50 mg/ml	2.1 %
1.00 mg/ml	0.61 %

These solutions were stored in glass-stoppered 1 ml micro-Erlenmeyer flasks and kept at below 0°.

An automatic pipette[35] was calibrated by weighing water to the nearest 0.02 mg and was found to deliver 4.400 ± 0.026 μl. at 25°. The statistical confidence limit is 95 %. This calibrated pipette was used throughout the series of experiments.

Three separate sheets of chromatographic paper (Schleicher & Schüll No. 2043 b) were spotted with eight equidistant spots of 4.400 ± 0.026 μl each, from the three glycine solutions. Each spot was formed by allowing the pipette to empty by capillarity. The chromatograms were developed in a descending manner for 24 hours with *n*-butanol–acetic acid–water, in the proportion of 4:1:5 and were then dried overnight (10 h) in an air current at 45°. The sheets were then cut along the direction of solvent flow into 3.8 cm wide strips, in such a way that the spots occurred centrally along the strips. Each paper strip was supported vertically and sprayed on both sides with a solution of ninhydrin (1 g of ninhydrin, 90 g of *n*-butanol and 10 g of phenol) until translucent, then immediately heated for 4 min in an oven saturated with water vapour, at $110 \pm 10°$.

Densitometry

The strips were left for 3 hours between sheets of clean filter paper and then passed through the photometer, using response No. 1 on the variable response recorder, and a slit aperture of 1×25 mm. The instrument was first zeroed on a blank area in the proximity of the spots. Each spot was passed ten times through the photometer and the area under each curve was measured ten times each with a manual planimeter.

The values are given in Table I and are the averages of about 800 area measurements. Some of the spots were also recorded at response setting No. 5, and these are given in Table II. It had been ascertained previously that ten photometric curves, with a total of one hundred area measurements, ten for each curve, would result in an error of 2.0 % with a 95 % confidence limit. It must be stated though, that these experiments were carried out with response setting No. 5 on the Varicord and then applied to response setting No. 1.

TABLE I

RESULTS OF MEASUREMENTS OF AREAS UNDER THE CURVES TRACED BY THE VARIABLE RESPONSE RECORDER, SETTING NO. 1

	0.10 mg/ml Glycine (7 spots, 10 curves, each 0.44 µg/spot)	*0.50 mg/ml Glycine (7 spots, 10 curves, each 2.20 µg/spot)*	*1.00 mg/ml Glycine 10 spots, 5 curves, each 4.40 µg/spot)*
No. of curves measured	70	83	50
Total No. of areas measured	700	830	500
$\bar{x}^{(+)}$	4.98 cm^2	15.29 cm^2	19.47 cm^2
$s_{\bar{x}}$	0.19	0.43	0.31
s	0.51	1.22	0.69
s'	0.47	1.14	0.62
δ	0.29	0.85	0.25
At 95 % confidence limit the error is	± 0.39 cm^2 or 7.8 %	± 0.86 cm^2 or 5.6 %	± 0.62 cm^2 or 3.2 %

+ $\bar{x}$ = arithmetic mean. $s_{\bar{x}}$ = mean error of mean (67 % confidence limit). s = standard error. s' = root mean square error. δ = mean deviation.

TABLE II

RESULTS OF MEASUREMENTS OF AREAS UNDER THE CURVES TRACED BY THE VARIABLE RESPONSE RECORDER SETTING NO. 5

	One spot of 0.10 mg/ml glycine	*One spot of 0.50 mg/ml glycine*	*One spot of 1.00 mg/ml glycine*
No. of curves measured	10	10	10
Total No. of areas measured	100	100	100
$\bar{x}$ (cm^2)	1.93	5.96	10.82
$s_{\bar{x}}^{(+)}$	0.03	0.05	0.14
s	0.09	0.14	0.46
s'	0.09	0.14	0.43
δ	0.08	0.11	0.34
At 95 % confidence limit the error is	± 0.06 cm^2 or 3.1 %	± 0.09 cm^2 or 1.5 %	± 0.29 cm^2 or 2.7 %

+ 67 % Confidence limit.

TABLE III

AREA MEASUREMENTS ON A SPOT OF 0.10 µg/ml GLYCINE AT VARIOUS TIME INTERVALS, RECORDER RESPONSE SETTING NO. 1

	0 h	*3 h*	*10 h*
No. of curves measured	9	10	10
Total No. of areas measured	90	100	100
$\bar{x}$ $(cm)^2$	4.23	4.39	4.19
$s_{\bar{x}}^{(+)}$	0.04	0.05	0.03
s	0.13	0.16	0.11
s'	0.12	0.15	0.11
δ	0.08	0.13	0.08

+ 67 % Confidence limit.

Determination of colour stability

To determine the variation of colour intensity of the spots after being sprayed with ninhydrin, one spot was passed through the photometer immediately after spraying and drying; the measurement was repeated after 3 hours and again after 10 hours. The results are given in Table III.

Determination of sensitivity to glycine concentration

Two chromatograms were spotted according to Table IV and were treated in the same way experimentally as the first three chromatograms. The results are given in Table V. All results have been plotted on the graph in Fig. 1.

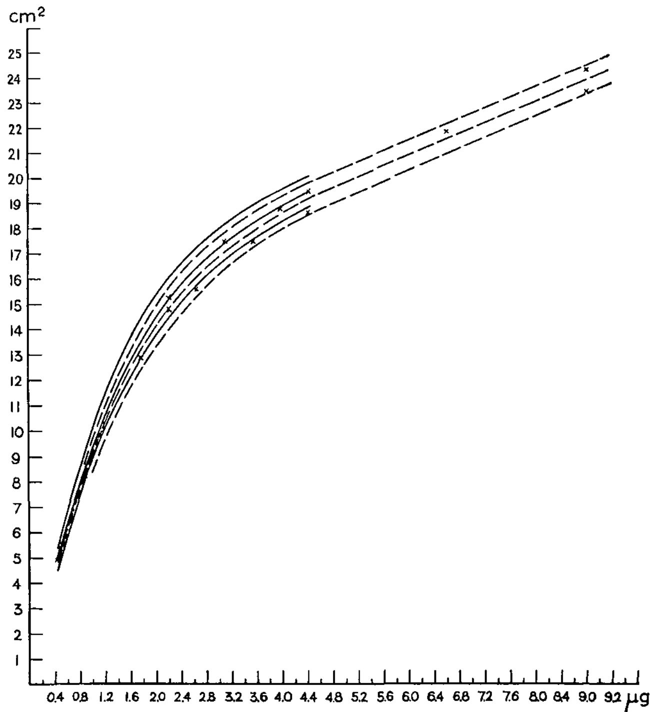

Fig. 1. Full line: Results of Table I. Dashed line: corrected curve for multiple spotting. See explanation in Discussion.

DISCUSSION AND RESULTS

The method can be made more precise if desired. For instance, the solutions of glycine can be prepared with much greater accuracy and the ninhydrin reaction can be made more uniform in the different spots by spraying the whole sheet of the chromatogram before cutting it into strips. Adherence to the experimental precautions given in the excellent work of HANES *et al.*[36], would undoubtedly result in smaller errors.

The method described in this paper was conducted in such a way, apart from not using filters with the photometer*, as to obtain the largest possible error and compare this with the 20 % limit previously given.

* There were none available in Argentina.

TABLE IV

GLYCINE CONCENTRATIONS EMPLOYED IN DETERMINATION OF SENSITIVITY OF THE METHOD

Spot No.	*No. of spottings with glycine solution of:*			*Amount of glycine spotted*	
	0.10 mg/ml	*0.50 mg/ml*	*1.00 mg/ml*	*µg*	*nmols. (10^{-9} mol)*
1	2	—	—	0.88 ± 0.04	5.86 ± 0.54
2	3	—	—	1.32 ± 0.06	17.6 ± 0.80
3	4	—	—	1.76 ± 0.08	23.4 ± 1.1
4	5	—	—	2.20 ± 0.09	29.3 ± 1.2
5	1	1	—	2.64 ± 0.08	35.2 ± 1.1
6	2	1	—	3.08 ± 0.09	41.0 ± 1.2
7	3	1	—	3.52 ± 0.10	46.9 ± 1.4
8	4	1	—	3.96 ± 0.12	52.7 ± 1.6
9	—	2	—	4.40 ± 0.12	58.6 ± 1.6
10	—	1	1	6.60 ± 0.11	87.0 ± 1.5
11	—	2	1	8.80 ± 0.17	117.2 ± 2.3
12	—	—	2	8.80 ± 0.11	117.2 ± 1.5

TABLE V

AREA MEASUREMENTS OF VARIOUS GLYCINE CONCENTRATIONS AS IN TABLE IV

Spot No.	*Recorder response setting No. 1. Mean area (95 % confidence limit) cm²*	*Recorder response setting No. 5. Mean area (95 % confidence limit) cm²*
1	8.28 ± 0.17	—
2	8.62 ± 0.17*	—
3	12.90 ± 0.34	—
4	14.80 ± 0.35	—
5	15.62 ± 0.29	—
6	17.49 ± 0.32	—
7	17.49 ± 0.49	7.34 ± 0.25
8	18.79 ± 0.24	—
9	18.63 ± 0.50	9.03 ± 0.18
10	21.85 ± 0.66	8.08 ± 0.21
11	24.31 ± 0.43	10.45 ± 0.17
12	23.42 ± 0.48	—

* Discarded.

The following sources of error contribute to the final result:

(a) weighing;
(b) volumetric: making up the solutions and pipetting;
(c) drying the chromatogram (volatilization of amino acids);
(d) the ninhydrin reaction (variability of colour response);
(e) variability of colour intensity with time;
(f) photometric;
(g) graphical: area measurement and proper base line.

Errors "a" and "b" can be calculated to a fair degree of accuracy; errors "c" and "e" can be supposed to have the same value for the test substance as for the unknown and are consequently not evaluated; errors "f" and "g" have been limited

to 2 % on the basis of experimental observation, as stated above. The remaining error "d" can be estimated by the difference between the overall error and the sum of the estimated errors. The values assigned to these are given in Table VI.

TABLE VI

CONTRIBUTION OF TYPES OF ERROR TO THE TOTAL ERROR

Kind of error	*% Error for solutions of glycine of concentration*		
	0.10 mg/ml	*0.50 mg/ml*	*1.00 mg/ml*
a	3.7	2.1	0.61
b	0.78	0.83	0.72
f + g	2.0	2.0	2.0
Total:	6.48	4.93	3.33

For the principal curve in Fig. 1, the per cent errors given in Table VII have been found:

TABLE VII

OVERALL ERRORS FOR VARIOUS GLYCINE CONCENTRATIONS

Area, cm²	*Concentration, μg*	*Error, %*
4.89	0.44 ± 0.05	11.3
15.29	2.20 ± 0.24	10.9
19.47	4.40 ± 0.56	12.7

From this it can be seen that if an unknown gave an area of 15.29 cm^2, the central full curve of Fig. 1 will give 2.20 μg of glycine, while the error limit curves will indicate an uncertainty range of 0.48 μg. Therefore, this area corresponds to 2.20 ± 0.24 μg glycine with a confidence limit of 95 %. The per cent error is 10.9 %. This means that for one nanomole of glycine, a quantity of 0.89 to 1.11 nanomoles will be found. This is sufficient accuracy for establishing that one instead of two nanomoles of glycine are bound to one nanomole of DNP-glycine.

By taking the difference between the overall error of 10.9 % and the estimated error of 6.5 % for the 0.10 mg/ml glycine solution, error "d" is calculated at 4.4 %.

The results of Table I have been plotted as already stated in the graph of Fig. 1. The central full line represents the most probable value of the area at the three experimental points. The upper and lower full lines represent the mean error of this value, with a confidence limit of 95 %. All experimental points should fall into the area determined by these last two curves. The points representing the data of Table V fall only into the lower half of this area and some are even below.

This can be explained by the fact that for the principal curve only one spotting was made, while for the points recorded in Table V a number of spottings were made at each spot on the paper. Consequently, a loss of glycine resulted.

If the whole curve is shifted parallel to itself by one half of the mean error of the mean value ($s_{\bar{x}}$-/2), as represented by the broken lines and is extrapolated with a

straight line from the point corresponding to 4.40 μg to the point at 8.80 μg, all experimental points fall within the error limits, well distributed in the upper and lower regions.

Evidently, the full curve will be valid even if various spottings are made in practical work; if conditions are more stringent, the overall error will be smaller.

TABLE VIII

OVERALL ERRORS FOR "WORKING CURVE"

Area, cm²	*Concentration, μg*	*Error, %*
8.28	0.88 ± 0.08	10.0
12.90	1.72 ± 0.18	10.5
14.80	2.20 ± 0.22	10.0
15.62	2.46 ± 0.24	9.9
17.49	3.28 ± 0.36	10.9
18.63	4.18 ± 0.48	10.9
21.85	6.96 ± 0.56	8.0

The per cent errors found for the broken line curve are given in Table VIII, and in this "working" curve the maximum error is 10.9 %.

ACKNOWLEDGEMENTS

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SUMMARY

A densitometric method, without the use of filters, for the determination of glycine after reaction with ninhydrin, is described and the overall error of the method is evaluated. The object of the study was to distinguish between DNP-Gly-Gly and DNP-Gly-Gly-Gly. The total spot density was determined by scanning through dry paper with a photometer and measurement of the area under the curve. The maximum error committed was 10.9 %, with a confidence limit of 95 %, which is about half the error admissible.

REFERENCES

[1] R. J. BLOCK, *Anal. Chem.*, 22 (1950) 1327.
[2] R. H. MÜLLER AND D. L. CLEGG, *Anal. Chem.*, 23 (1951) 403.
[3] A. R. PATTON AND P. CHISM, *Anal. Chem.*, 23 (1951) 1683.
[4] E. F. MCFARREN AND J. A. MILLS, *Anal. Chem.*, 24 (1952) 650.
[5] R. C. SALANDER, M. PIANO AND A. R. PATTON, *Anal. Chem.*, 23 (1953) 1252.
[6] J. F. ROLAND AND A. M. GROSS, *Anal. Chem.*, 26 (1954) 502.
[7] J. C. UNDERWOOD AND L. B. ROCKLAND, *Anal. Chem.*, 26 (1954) 1553.
[8] L. B. ROCKLAND AND J. C. UNDERWOOD, *Anal. Chem.*, 28 (1956) 1679.
[9] A. LACOURT, *Mikrochim. Acta*, (1955) 824.
[10] A. LACOURT AND P. HEYNDRYCKX, *Mikrochim. Acta*, (1956) 1211.
[11] A. LACOURT AND P. HEYNDRYCKX, *Mikrochim. Acta*, (1956) 1389.
[12] A. LACOURT AND P. HEYNDRYCKX, *Mikrochim. Acta*, (1956) 1621.
[13] H. C. EHRMANTRAUT AND A. WEINSTOCK, *Biochim. Biophys. Acta*, 15 (1954) 589.
[14] J. M. MILLER AND L. B. ROCKLAND, *Arch. Biochem. Biophys.*, 40 (1952) 416.
[15] R. R. REDFIELD AND E. S. G. BARRON, *Arch. Biochem. Biophys.*, 35 (1952) 443.
[16] A. BEVENUE AND K. T. WILLIAMS, *Arch. Biochem. Biophys.*, 73 (1958) 291.

[17] R. A. Boissonnas, *Helv. Chim. Acta*, 33 (1950) 1966.
[18] S. V. Vaeck, *Anal. Chim. Acta*, 10 (1954) 48.
[19] C. Bergamini and W. Versorese, *Anal. Chim. Acta*, 10 (1954) 328.
[20] S. V. Vaeck, *Anal. Chim. Acta*, 12 (1955) 443.
[21] R. L. Wieme, *J. Chromatog.*, 1 (1958) 166.
[22] A. Bevenue and K. T. Williams, *J. Chromatog.*, 2 (1959) 199.
[23] B. M. Johnstone and G. P. Briner, *J. Chromatog.*, 2 (1959) 513.
[24] R. B. Ingle and E. Minshall, *J. Chromatog.*, 8 (1962) 369.
[25] H. B. Bull, J. W. Hahn and V. H. Baptist, *J. Am. Chem. Soc.*, 71 (1949) 550.
[26] L. B. Rockland and M. S. Dunn, *J. Am. Chem. Soc.*, 71 (1949) 4121.
[27] R. J. Block, *Science*, 108 (1948) 608.
[28] L. S. Fosdick and R. Q. Blackwell, *Science*, 109 (1949) 314.
[29] S. V. Vaeck, *Nature*, 172 (1953) 213.
[30] A. Lacourt and P. Heyndryckx, *Nature*, 176 (1955) 880.
[31] D. Bolling, H. A. Sober and R. J. Block, *Federation Proc.*, 8 (1949) 185.
[32] A. R. Neurath and F. Fric, *Chem. Zvesti*, 13 (1959) 247.
[33] G. Kortüm and J. Vogel, *Angew. Chem.*, 71 (1959) 451.
[34] P. Albers and R. Freiskorn, *Ann.*, 622 (1959) 150.
[35] M. R. E. Jellinek, *Rev. Asoc. Arg. Químicos y Técnicos Ind. Cuero*, 2 (1961) 96.
[36] C. S. Hanes, *et al.*, *Can. J. Biochem. Physiol.*, 33 (1955) 416; 39 (1961) 119; 39 (1961) 141; 39 (1961) 163; 39 (1961) 417; 39 (1961) 427; 39 (1961) 439.
[37] R. J. Block, E. L. Durrum and G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New York, 1958.
[38] Ch. H. Orr, *Anal. Chem.*, 33 (1961) 158.

A NEW METHOD FOR QUANTITATIVE PAPER CHROMATOGRAPHY

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The high efficiency of paper chromatography in qualitative work explains the numerous attempts to find methods for quantitative estimation of small samples separated by this technique.

Visual comparison[1] of the colour of the spot, allows semi-quantitative analysis, and other authors have carried out spot size measurements[2] or direct colorimetric determinations[3-10].

More accurate results are obtained by making colorimetric measurements after elution[11-12], and "retention analysis" is a good method of quantitative estimation without elution[13-14].

In the present work an indirect quantitative determination is described: the spots are detected by iodine vapour[15-16], eluted, and the amount of fixed halogen is determined.

Iodine vapour was first used by BRANTE[15] to detect nitrogenous substances on paper chromatograms. At a later date, MARINI-BETTOLO AND GUARINO[16] showed the possibilities of iodine as a detector of non-nitrogenous substances.

In our experiments we have established the proportionality between the amount of substance and the iodine fixed by the spot.

The knowledge of the amount of iodine fixed by a certain quantity of substance enables us to give a numerical value to the sensitivity, which is defined as the number of μg of iodine fixed by one μg of substance, and values for various substances are given in Table I. These values vary greatly with experimental conditions, for example in the case of the amino acids, the sensitivity is greater if the chromatogram is dried at 160° (toasting) before being exposed to the iodine vapours.

In general we have noticed that the presence of moisture in the iodine chamber increases the sensitivity but that too high a humidity causes spot diffusion. The work was therefore carried out in an atmosphere at 76 % relative humidity stabilized with a saturated solution of KCl.

In some cases (benzedrine, atropine, etc.), the colour sensitivity improves if the chromatogram is left some minutes in the air after the exposure. In this way the paper-fixed iodine is rapidly lost and the contrast between the spot and the paper increases.

EXPERIMENTAL

Apparatus and reagents

Detection chamber. An ordinary desiccator of 15–20 cm diameter, with two small dishes at the bottom, one for the iodine crystals and the other for the KCl saturated solution, makes a good chamber.

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TABLE 1

SENSITIVITY OF COMPOUNDS TO IODINE VAPOUR

Substance	*Sensitivity (in μg)*	*Solvent*	*Drying temperature (°)*	*Exposure (h)*
Alanine	2.1	Butanol–acetic acid	160	24
Arginine	1.8	Butanol–acetic acid	160	24
Glycine	1.9	Ethanol–NH_4OH	160	24
Tryptophan	1.9	Ethanol–NH_4OH	160	24
Leucine	1.8	Ethanol–NH_4OH	160	24
Glucose	0.5	Ethanol–NH_4OH	160	24
Pyramidon	5.6	Butanol–acetic acid	120	3
Creatinine	3.7	Butanol–acetic acid	120	3
Ephedrine	1.8	Butanol–acetic acid	120	5
Sulphathiazole	1.6	Butanol–acetic acid	120	18
Benzedrine	1.6	Butanol–acetic acid	120	18
Atropine	4.3	Butanol–acetic acid	120	18
Brucine	2.9	Butanol–acetic acid	120	18
Novocaine	4.6	Butanol–acetic acid	120	18
Pilocarpine	1.9	Butanol–acetic acid	120	18
Codeine	1.7	Butanol–acetic acid	120	18
Malonic acid	2.0	Ethanol–NH_4OH	120	2
p-Aminobenzoic acid	1.4	Ethanol–NH_4OH	120	5
Ascorbic acid	2.7	Butanol–acetic acid	120	2
Thiamine–HCl	0.5	Propanol–HCl	80	20

Stand for the paper cut-outs. A grill of a size suited to the desiccator was made with 4 mm diameter glass rod (Fig. 1).

Micropipettes. These can be easily made with capillary tubes marked so that they deliver 0.005 ml. If the same pipette is used for the sample solution and the standard solution, it is unnecessary to know the exact volume used.

Chromatographic paper. This should be large enough for duplicates of the sample solution together with three or four spots of the reference solution to be chromatographed.

Before spotting the paper is divided up into 2 cm wide strips, marked in pencil, which run perpendicular to the line along which the samples are placed.

The paper must be handled by touching only the edges with clean fingers.

5–10 % KI solution.

1 % soluble starch solution.

0.001 N (approx.) sodium thiosulphate. Prepared by dilution of a more concentrated solution.

Standard reference solutions. 0.5–5 % of the substance in an appropriate solvent. The concentration range chosen must be near that of the sample solution and all solutions must be freshly prepared.

EEL or a similar type photocolorimeter. A calibration curve is made with a solution of iodine in 5 % KI.

Procedure

When preparing the chromatogram special care must be taken in measuring the sample volume, which must be placed at the starting line and equidistant from the guide lines.

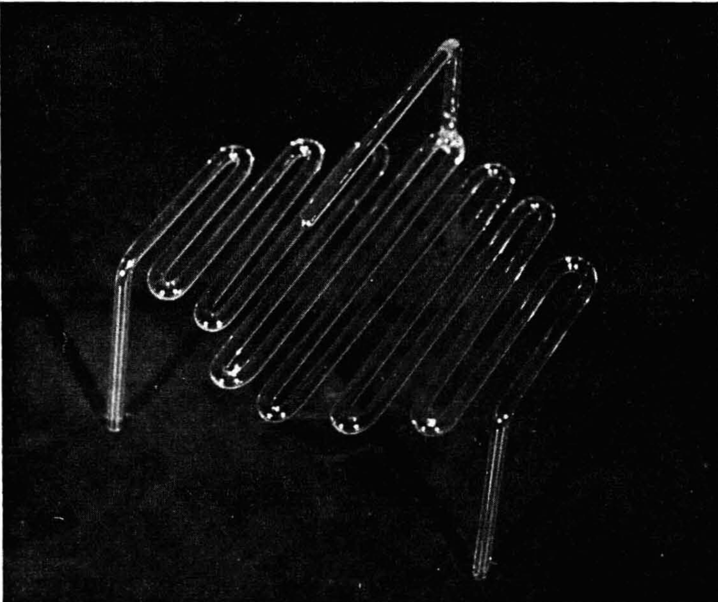

Fig. 1. Stand for the paper cut-outs.

After the chromatogram has been developed the solvent is removed by heating in an oven. The temperature used varies with the solvent mixtures and with the substance; for example propanol–HCl mixture must not be heated to more than 80°; on the other hand, to sensitize some substances like alanine, one must reach 160°. Usually the chromatogram is heated for 15–20 min at 120°.

When dry the chromatogram is placed in the iodine chamber for the qualitative detection of the spots on the paper. After their appearance, the chromatogram is removed from the iodine chamber, placed on a clean surface and two parallel lines are drawn with a pencil near the upper and lower edges of the spots. In this way one obtains a series of identical squares, which are properly numbered, cut out and finally placed back on the glass stand in the iodine chamber for the completion of the reaction.

These cut-outs are best handled with metal tweezers. The exposure time varies from 2–20 h depending on the sensitivity and the size of the sample. When all the spots stand out clearly against the paper the reaction is usually complete.

Each spot is individually eluted from the paper by KI solution contained in 50–100 ml beakers.

For the iodometric titration, the elution is carried out with 5 ml of 10 % KI, three drops of 1 % starch solution are then added and the titration is carried out with 0.001 *N* (approx.) sodium thiosulphate.

The elution for the colorimetric measurement is made with exactly 10 ml of 5 % KI, and the colour is developed with five drops of 1 % starch solution.

Either the thiosulphate or the colorimetric data enable us to calculate in a relative way the μg of iodine fixed in each cut-out by comparison with the calibration curve drawn for the series of reference samples.

RESULTS

In order to illustrate the scope of this method some results are given in Table II and Fig. 2. In alle cases ascending chromatograms on Whatman No. 1 paper were run and development times varied between 60 and 150 min.

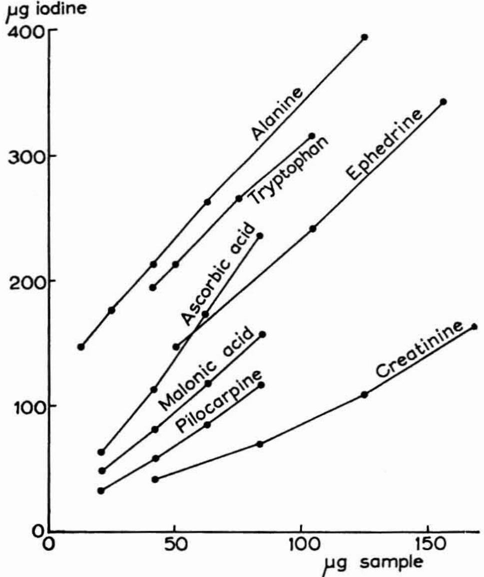

Fig. 2. Examples of calibration curves. Each point is an average of two spots.

The number of µg of fixed iodine and sample indicated are not absolute values and they serve only for comparison purposes.

DISCUSSION

As this method allows the determination of very different substances due to the lack of specificity of iodine vapour, great care has to be taken over the chromatographic separation to avoid interferences. However, it has the advantage that the apparatus and the reagents employed are in common use.

The reproducibility of the results is very good and in the duplicate determinations the normal deviation from the mean is less than 5 %. The best results correspond to the more sensitive substances and the sensitivity has to be more than 1 in order to obtain good results with small samples.

Thin-layer chromatography offers new possibilities for this method.

ACKNOWLEDGEMENT

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TABLE II

RESULTS

Substance	*µg of*		*Solvent*	*Drying*	*Exposure (h)*	*Method*
	Sample	*Iodine*				
Alanine	13	151	Butanol–acetic acid	30 min at 160°	20	Iodometric
	25	178				
	42	215				
	63	266				
	126	392				
Tryptophan	42	198	Ethanol–NH_4OH	20 min at 160°	20	Colorimetric
	51	216				
	75	267				
	105	315				
Ascorbic acid	21	66	Butanol–acetic acid	15 min at 120°	2	Colorimetric
	42	116				
	63	174				
	84	237				
Creatinine	42	45	Butanol–acetic acid	15 min at 120°	2	Colorimetric
	84	73				
	126	110				
	168	163				
Malonic acid	21	50	Ethanol–NH_4OH	15 min at 120°	5	Colorimetric
	42	83				
	63	119				
	84	158				
Pilocarpine	21	35	Butanol–acetic acid	15 min at 120°	3	Colorimetric
	42	60				
	63	87				
	84	118				
Ephedrine	52	149	Butanol–acetic acid	15 min at 120°	5	Iodometric
	105	242				
	157	342				

SUMMARY

A general method is described which allows a quantitative estimation of those substances that are detectable by iodine vapour on paper chromatograms. The quantity of iodine fixed by the substance is determined after elution with KI solution, either by iodometry or colorimetry.

REFERENCES

[1] K. Miyaki, K. Satake and M. Hayashi, *J. Pharm. Soc. Japan*, 71 (1951) 249.
[2] R. Fisher, K. Parson and G. Morrison, *Nature*, 161 (1948) 764.
[3] B. M. Bull, J. W. Hahn and V. H. Baptist, *J. Am. Chem. Soc.*, 71 (1949) 550.
[4] R. Fisher and R. Holmes, *Biochem. J.*, 44 (1949) 54.
[5] L. S. Fosdick and R. Q. Blackwell, *Science*, 109 (1949) 314.
[6] R. H. Mueller and D. L. Clegg, *Anal. Chem.*, 21 (1949) 1123.
[7] R. J. Block, *Anal. Chem.*, 22 (1950) 1327.
[8] A. Lacourt, *Mikrochim. Acta*, (1957) 269.
[9] H. C. Ehrmantraut and A. Weinstock, *Biochim. Biophys. Acta*, 15 (1954) 589.
[10] H. R. Roberts, *Anal. Chem.*, 29 (1957) 1443.
[11] A. Flood, E. Hirst and J. Jones, *Nature*, 160 (1947) 86.
[12] L. Naftalin, *Nature*, 161 (1948) 763.
[13] Th. Wieland and E. Fischer, *Naturwiss.*, 35 (1948) 29.
[14] E. R. Reichl and J. E. Löffler, *Mikrochim. Acta*, (1954) 226.
[15] G. Brante, *Nature*, 163 (1949) 651.
[16] G. B. Marini-Bettolo and S. Guarino, *Experientia*, 6 (1950) 309.

SEPARATION OF CYSTEINE AND ITS OXIDATION PRODUCTS BY PAPER AND THIN-LAYER CHROMATOGRAPHY

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Many workers have attempted to identify free cysteine by paper chromatographic methods[1,2], but usually it is indistinguishable from the principal oxidation product, cystine. During a study of the products of reaction of tobacco smoke condensate with cysteine, it has been necessary to assess the amount of breakdown of cysteine itself during the procedure for chromatography. Whilst the chromatogram is running, cysteine does not oxidise appreciably, as shown by a well defined spot with little "tailing". The major breakdown of cysteine appears to occur whilst the material is standing at the origin of the chromatogram during equilibration prior to development, and also on a two-dimensional run in the period between removal of the first solvent and re-development with the second component. Oxidation is particularly noticeable in the latter case, when papers are allowed to stand for an appreciable time before the second development stage. In this connection it is advantageous to use the technique of thin-layer chromatography, where a full two-dimensional separation can be achieved in one working day.

The breakdown of cysteine after standing on the origin of a paper chromatogram was first noticed by TOENNIES AND KOLB[3], but they did not identify the oxidation products produced. Similar patterns of products are formed during the breakdown of methionine[4,5] and other amino acids[6,7], the multiplicity of spots produced on chromatography were readily shown by autoradiographic techniques, but again only a tentative identification of a few of the products was made.

In the present work we have attempted to elucidate the nature of the breakdown products obtained during the procedure for chromatography of mixtures containing cysteine labelled with sulphur-35. The oxidation of cysteine, when placed for long periods at the origin of chromatograms, has provided an accelerated test, and has also indicated a possible mechanism for the degradation process.

EXPERIMENTAL

Preparation of [^{35}S]-cysteine hydrochloride

A convenient preparation of microgram amounts of fresh [^{35}S]-cysteine hydrochloride, suitable for an autoradiographic investigation, was achieved by means of the disulphide exchange reaction[8]. Cysteine hydrochloride (0.33 μmole) was spotted at the origin of the chromatogram, followed immediately by [^{35}S]-cystine hydrochloride (1 μC, 0.033 μmole) (Radiochemical Centre, Amersham, Bucks. England). The mixture

was exposed to ammonia vapour for one minute to complete equilibration before acidifying with hydrogen chloride. Approximately 80 % of the radioactivity in the cystine is located in the cysteine after this equilibration process.

[^{35}S]-Cysteine hydrochloride was found to undergo appreciable radiolysis on storage, even in acid solution at temperatures near 0°, whereas [^{35}S]-cystine hydrochloride stored under nitrogen, was considerably more stable over long periods under the same conditions[9,10]. Reduction of [^{35}S]-cystine hydrochloride with tin and hydrochloride acid was found to be wasteful of material, particularly on a micro-scale, with no guarantee of complete freedom from metal and other impurities.

Chromatography and autoradiography

The technique of ascending chromatography was used throughout this work, except where streaking of material was necessary as a prelude to isolation, when a descending method was employed. Three main solvent systems were employed[11], as with slight modification they were also applicable to thin-layer plates.

Mixture 1: Butanol–acetic acid–water (120:30:50, v/v/v)

Mixture 2: Butanol–pyridine–water (1:1:1, v/v/v)

Mixture 3: Phenol–water (4:1, w/v).

The method of BRENNER AND NIEDERWIESER[12] was most satisfactory for thin-layer chromatography, using unactivated Kieselgel-G plates and the above solvents with lower water contents.

Radioactive substances on the chromatograms were located by autoradiography using "Kodirex X-ray film" (Kodak Limited, England), exposed for periods of up to 48 h. The film was protected from acidic materials on the papers and plates by thin polythene sheets. Parallel series of experiments were performed using labelled and unlabelled cysteine to detect any appreciable concurrent radiolytic breakdown of starting materials.

Oxidative breakdown of cysteine on chromatography paper

Figs. 1 and 2 illustrate the decomposition of cysteine hydrochloride on standing at the origin of a paper chromatogram for increasing periods of time. Even after 100 h, a small quantity of cysteine remained, but the number of degradation products and their concentration usually increased with time.

When acid-washed and hardened paper (Whatman No. 540) was used, either untreated or washed with a solution of oxalic acid, a slight decrease in the extent of breakdown of cysteine was observed. In contrast, a significant decrease in both oxidation and degradation was found when the procedure for chromatography was carried out with exclusion of light. The disappearance of cysteine was accelerated by both ultra-violet irradiation and the addition of metal ions to the cysteine solution. For example, the presence of 0.3 μmole Fe^{2+} catalysed the removal of all the cysteine in 48 h, compared with 81 h for 0.03 μmole Fe^{2+}. A little breakdown of cysteine occurred during the actual chromatographic development, as shown by a slight streaking superimposed on the discrete spots due to the degradation products. Variations in the moisture content of the paper did not appear to be a critical factor

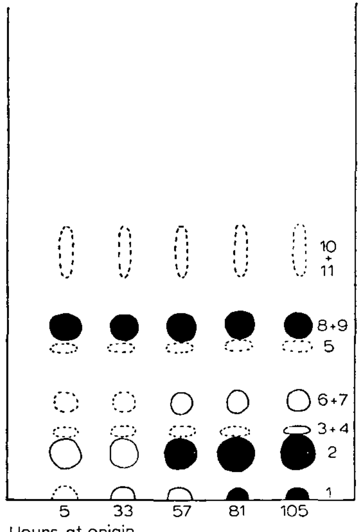

Fig. 1. Composite one-dimensional chromatogram and autoradiograph of cysteine hydrochloride and its degradation products. Solvent mixture 1. Whatman No. 1 paper. Identification of spots: 1 = cystine (unionised); 2 = cystine (ionised); 3 = cysteic acid, cystine disulphoxide and cystine monosulphoxide; 4 = β,β'-diamino-β-carboxydiethyl disulphide; 5 = cysteine sulphinic acid; 6 = cystamine and cysteine sulphoxylic acid; 7 = taurine; 8 = cysteine; 9 = unknown; 10 = β-aminoethylsulphinic acid; 11 = cysteamine. Approximate concentration in spot area: strong = solid circle; medium = continuous circle; weak = broken circle.

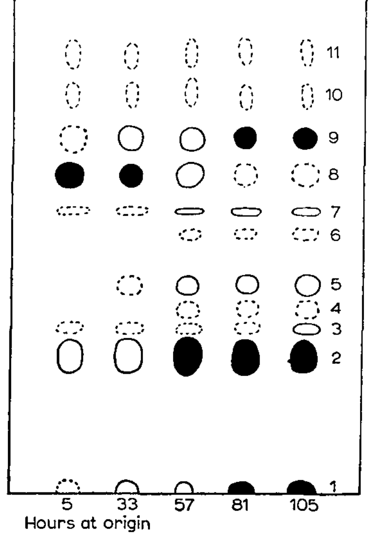

Fig. 2. Composite one-dimensional chromatogram and autoradiograph of cysteine hydrochloride and its degradation products. Solvent mixture 2. Whatman No. 1 paper. For key to spot numbers and symbols, see legend to Fig. 1.

in the oxidation process, as indicated by a comparison between papers hung in dry and water-saturated atmospheres.

In previous studies of the chromatography of cysteine and derivatives[1,2], low R_F values were quoted for cysteine itself, probably due to the rapid oxidation to cystine. In the present work, the R_F values for cysteine appear to be consistent with the structural relationships to the major oxidation products and other amino acids. Identification of the products from chromatography was made either from the R_F values, or by superposition of authentic ninhydrin-positive materials on radioactive cysteine at the origin, followed by comparison of the resulting chromatogram and autoradiograph. A sample of β,β'-diamino-β-carboxydiethyl disulphide was prepared by the exchange reaction between cystine and cystamine.

For two-dimensional chromatography, the preferred order of developing solvents was mixture 1 followed by mixture 2. Mixture 3 was not used extensively, as even redistilled phenol caused some breakdown of cysteine during the development[6,7]. The use of solvent mixtures 1 and 2 were also dictated by the requirements of a study of the reaction between cysteine and tobacco smoke condensate.

An example of an autoradiograph from a two-dimensional chromatogram is shown in Fig. 3. Here the [^{35}S]-cysteine hydrochloride was prepared on the origin of the chromatogram immediately prior to chromatography. Slightly differing results were obtained (Fig. 4) when oxalic acid-washed Whatman No. 540 paper was used.

The spots due to taurine (in Fig. 3) are now absent, even though the starting material had stood at the origin for 81 h before development. It is probable that the oxidations of cysteamine sulphinic acid to taurine, and cysteine sulphinic acid to cysteic acid, are catalysed by metal impurities in the paper. If untreated Whatman No. 1 paper is used, more extensive decomposition of the cysteine occurs.

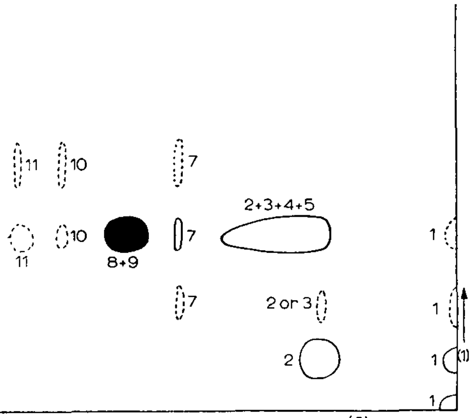

Fig. 3. Composite two-dimensional chromatogram and autoradiograph of cysteine hydrochloride after application to origin immediately prior to development. Whatman No. 540 paper. For key to spot numbers and symbols, see legend to Fig. 1.

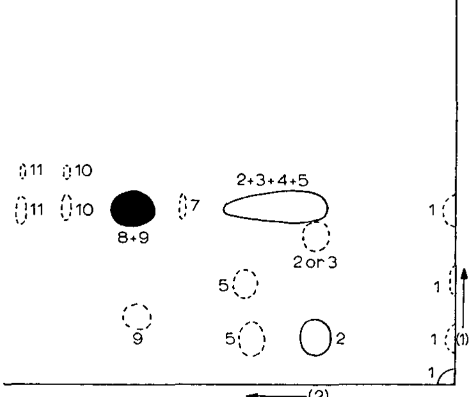

Fig. 4. Composite two-dimensional chromatogram and autoradiograph of cysteine hydrochloride after being allowed to stand at origin for 81 h before development. Whatman No. 540 paper washed with *M* oxalic acid. For key to spot numbers and symbols, see legends to Fig. 1.

When [^{35}S]-cysteine hydrochloride is spotted at the origin, treated with ammonia and allowed to stand for a long period of time, a different pattern of oxidation and degradation products is formed containing a considerable amount of fast-moving material. This result is due to the enhanced radiolytic decomposition of cysteine in alkaline solution, and details of the products will be reported in a subsequent communication.

Thin-layer chromatography of cysteine and its degradation products offers the advantages of speed of operation and high capacity of the stationary phase. A two-dimensional separation can be achieved over the period of one working day, thus lessening the inherent decomposition of the cysteine mixture. The separation of products is comparable to that obtained from paper chromatography over an equivalent solvent run.

Mechanism of oxidation of cysteine during the procedure for chromatography

The general mechanism of the oxidation of cysteine to cystine has been discussed in detail by TARBELL[13], the reaction probably being free radical in character and catalysed by metal ion impurities. In the present study this reaction occurs together with another oxidation and decarboxylation sequence, the first stage of which is the conversion of cysteine to cysteamine. Subsequently the cysteamine is oxidised to cysteamine sulphinic acid and then to taurine. It has been suggested that in biological systems, cysteine sulphinic acid is produced initially from cysteine followed by

decarboxylation and further oxidation to taurine[14]. This reaction sequence may also occur in the oxidation of cysteine on chromatography paper. The present results exclude the formation of taurine by decarboxylation of cysteic acid or cystine disulphoxide. No evidence was obtained for a progressive decomposition of cysteic acid or cystine disulphoxide when spotted onto chromatography paper and allowed to stand for long periods of time before development. The trace of cystamine observed is formed by the oxidation of cysteamine, whilst β,β'-diamino-β-carboxydiethyl disulphide is formed by the disulphide exchange reaction between cysteamine or cystamine and cysteine or cystine.

The metal-catalysed oxidations of cysteine on chromatography paper are decreased by using Whatman No. 540 paper washed with complexing agents. The overall oxidation is minimised by decreasing the time required for the process of chromatography, the latter preferably carried out with the exclusion of light.

ACKNOWLEDGEMENTS

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SUMMARY

The nature of the decomposition products formed from cysteine during the procedure for chromatography has been investigated. Concurrent oxidation and decarboxylation processes have been shown to occur when cysteine, either free or as the hydrochloride, is finely dispersed on chromatography paper and allowed to stand for extended periods of time. The total amount of decomposition is reduced by purification of chemicals and chromatography paper, and by exclusion of light.

REFERENCES

1 P. J. Peterson and G. W. Butler, *J. Chromatog.*, 9 (1962) 70.
2 J. Fondari and C. Richert, *J. Chromatog.*, 9 (1962) 262.
3 G. Toennies and J. J. Kolb, *Nature*, 177 (1956) 281.
4 J. Kolousek, J. Liebster and A. Babicky, *Nature*, 179 (1957) 521.
5 J. Kopoldova, J. Kolousek, A. Babicky and J. Liebster, *Nature*, 182 (1958) 1074.
6 A. K. Huggins and V. Moses, *Nature*, 191 (1961) 668.
7 V. Moses, *J. Chromatog.*, 9 (1962) 241.
8 J. Greenstein and M. G. Winitz, *Chemistry of the Amino Acids*, Vol. 2, Wiley, New York, 1961, p. 1618.
9 J. C. Fletcher and A. Robson, *Biochem. J.*, 84 (1962) 439.
10 J. R. Ogle, personal communication, The Radiochemical Centre, Amersham, Bucks. England.
11 I. Smith, *Chromatographic and Electrophoretic Techniques*, Vol. 1, Heinemann, London, 1960, p. 84.
12 M. Brenner and A. Niederwieser, *Experientia*, 16 (1960) 378.
13 D. S. Tarbell, in N. Kharasch (Editor), *Organic Sulphur Compounds*, Vol. 1, Pergamon, London, 1961, p. 97.
14 J. Awapara and W. J. Wingo, *J. Biol. Chem.*, 203 (1953) 189.

SÉPARATION DE LA SOLANINE, DE LA CHACONINE ET DE LA SOLANIDINE PAR CHROMATOGRAPHIE SUR COUCHES MINCES

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La solanine, la chaconine et la solanidine sont les principaux alcaloïdes présents dans les solanacées, particulièrement dans la pomme de terre (*Solanum tuberosum*). En plus d'un noyau aglycone, la solanidine, la solanine comprend une molécule de chacun des hydrates de carbone suivants: glucose, galactose et rhamnose. La chaconine ne diffère de la solanine que par l'absence de galactose[1-3].

Le point de fusion a servi longtemps de critère principal pour mesurer le degré de pureté de ces substances. Cependant, dans le cas de la solanine, plusieurs auteurs ont rapporté différents points de fusion, soit de 235° à 285° environ, démontrant alors que certaines préparations n'avaient été que partiellement purifiées[2]. Heureusement, les techniques plutôt récentes de séparation par chromatographie sur papier et sur colonne ont permis de déceler dans les extraits de solanine la présence de la chaconine et de quelques composés contenant le même aglycone[4,5]. La présence de ces substances analogues peut expliquer en partie la variation dans les points de fusion rapportés pour la solanine[1,6]. Cependant, nous avons pu constater que la chromatographie sur papier ne permet pas de séparer la solanidine de la solanine et de la chaconine. D'autre part, il semble définitivement établi que la solanidine existe à l'état libre avec les deux autres alcaloïdes, atteignant jusqu'à 33 % de la quantité totale des trois alcaloïdes dans certaines variétés de pommes de terre[3]. Nous avons donc eu recours à la chromatographie sur couches minces pour effectuer une séparation plus complète. Cette méthode nous a aussi permis de vérifier le point de fusion et le degré de pureté de nos extraits. Ce sont les résultats de ces travaux que nous voulons présenter ici.

MATÉRIEL ET MÉTHODES

Préparation des alcaloïdes

La méthode de BAKER *et al.*[7] fut généralement utilisée pour l'extraction des alcaloïdes de tubercules de pommes de terre conservés en caveau à 4° ou lyophilisés. Deux à dix grammes de tubercules moulus finement sont extraits dans un appareil à extraction continue durant 16 à 18 h avec du méthanol contenant 2 % d'acide acétique. Le méthanol est enlevé dans un évaporateur rotatif sous le vide à une température

inférieure à 30°. Après l'addition de 3 ml d'une solution d'acide sulfurique à 1 %, le résidu est filtré et le filtre lavé avec 3 autres ml de la même solution d'acide sulfurique. Le filtrat est alors alcalinisé avec de l'ammoniaque. Les alcaloïdes précipitent complètement à une valeur pH de 10.0 environ. Après avoir été refroidi, le précipité est enlevé par centrifugation ou par filtration et lavé plusieurs fois avec une solution ammoniacale à 1 %. A ce stade, la solanidine peut être séparée des deux autres alcaloïdes par extraction avec de l'acétone dans un extracteur Soxhlet durant 15 à 16 h. La solanine et la chaconine sont ensuite extraites du résidu à chaud avec de l'éthanol à 80 % d'où la solanine crystallise par refroidissement[8]. Pour la chromatographie, les échantillons de solanidine ou de solanine sont généralement solubilisés dans de l'éthanol contenant 1 à 2 % d'acide acétique.

La solanine et la chaconine furent séparées et purifiées par chromatographie sur colonne d'oxyde d'aluminium (Al_2O_3) suivant les techniques de PASESHNICHENKO ET GUSEVA[6].

Les points de fusion furent déterminés avec l'appareil de Fisher-Johns calibré avec un sel pur de phénolphthaléine.

Des échantillons de solanidine et de solanine nous ont été fournis gratuitement par les Laboratoires Lyons de Londres et le Dr. A. ZITNAK du Collège Agricole de Guelph, Ontario.

Chromatographie sur couches minces

Les techniques de chromatographie sur couches minces furent utilisées pour séparer les trois alcaloïdes et pour vérifier leur degré de pureté après chaque essai de purification. Les plaques de verre de 20 cm × 20 cm sont recouvertes d'une couche mince de silica gel G et développées par voie ascendante pendant deux heures jusqu'à une hauteur de 12 à 15 cm environ dans des jarres contenant différents solvants[9]. Après le séchage des plaques à l'air libre, les alcaloïdes sont révélés par la pulvérisation d'une solution saturée de trichlorure d'antimoine dans le chloroforme (réactif de Carr-Price). Les alcaloïdes apparaissent avec une coloration mauve qui change au violet après chauffage pendant quelques minutes à 70°. Le réactif de Marquis peut aussi être pulvérisé sur les plaques de verre[10]. Malheureusement, la coloration pourpre à violet des taches disparaît rapidement et les plaques sèchent difficilement.

Dosage des alcaloïdes

Les alcaloïdes furent dosés par colorimétrie avec un spectrophotomètre Beckmann, modèle DU, à une longueur d'onde de 570 mμ suivant la méthode de BAKER *et al.*[7].

Hydrolyse des alcaloïdes

La solanine et la chaconine ont été hydrolysées dans une solution *N* d'acide chlorhydrique suivant les indications de ROOKE *et al.*[11]. Pour la séparation des hydrates de carbone de la solution hydrolysée, nous nous sommes servis de la chromatographie sur papier à deux dimensions en utilisant comme solvants du phénol saturé d'eau dans la première direction et un mélange de butanol–acide acétique–eau (4:1:5, phase supérieure) dans la deuxième direction, et comme révélateur, le phthalate acide d'aniline[12]. Les taches des hydrates de carbone prennent alors une coloration brune.

RÉSULTATS ET DISCUSSION

Dosage par colorimétrie des alcaloïdes

La Fig. 1 montre que les courbes obéissent à la loi de Bougueur et Beer du moins aux concentrations de 0 à 40 μg par ml pour la solanidine, et de 0 à 125 μg par ml pour la solanine. On constate de plus que la densité optique de la solanidine à des

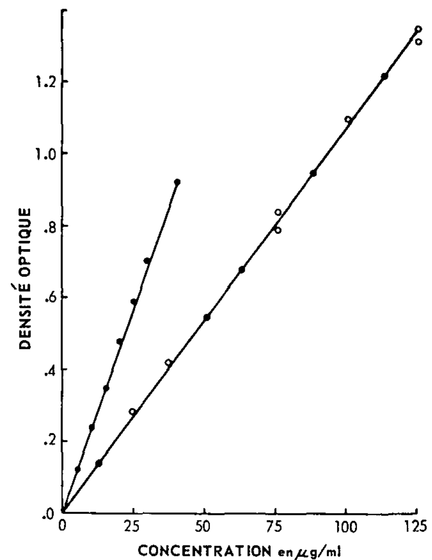

Fig. 1. Variation de la densité optique de la solanine et de la solanidine en fonction de la concentration exprimée en μg/ml de solution colorée après traitement avec le réactif de Marquis[7]. O—O Solanine; $y = 0.01085x$. ●—● Solanidine; $y = 0.02337x$.

concentrations égales est plus de deux fois plus grande que celle de la solanine. Ceci démontre que la portion aglycone de la solanine et de la chaconine, la solanidine, réagit seule avec le réactif de Marquis, indépendamment de la présence des hydrates de carbone.

Chromatographie sur couches minces

Le Tableau I donne les différents solvants utilisés pour la séparation des trois alcaloïdes sur les plaques de verre recouvertes de silica gel, les valeurs de R_F correspondantes et la forme des taches pour chacun des alcaloïdes. C'est un mélange d'une partie d'acide acétique et de trois parties d'éthanol à 95 % qui a donné les meilleurs résultats avec des valeurs de R_F de 0.22, 0.54 et 0.62 respectivement pour la solanine, la chaconine et la solanidine. Si toutefois on a observé des valeurs de R_F différentes d'un chromatogramme à l'autre pour une même substance, cela n'a pas eu de conséquence puisque le rapport des mobilités des taches demeurait constant. La chromatographie sur couches minces nous a permis de déceler des quantités aussi minimes que 1 μg de solanidine et 2 μg de solanine ou de chaconine.

La chromatographie sur couches minces nous a aussi aidés à vérifier rapidement le degré de pureté des alcaloïdes à chacune des étapes de leur purification. C'est ainsi

TABLEAU I

VALEURS DE R_F ET FORME DES TACHES DE LA SOLANINE, DE LA CHACONINE ET DE LA SOLANIDINE OBTENUES AVEC DIFFÉRENTS SOLVANTS

*Solvant**	*Concentration*	*Solanine*		*Chaconine*		*Solanidine*	
		R_F	*Tache*	R_F	*Tache*	R_F	*Tache*
$CHCl_3$–AcOH–BuOH	5:1:4	0	—	0	—	—	allongé
$CHCl_3$–AcOH–MeOH	85:2:13	0	—	0	—	0.51	ovale
AcOH–EtOH 95 %							
	3:1	0.1	allongée	0.2	allongée	0.80	ronde
	1:1	0.21–0.22	ovale	0.56	ovale	0.63–0.65	ovale
	1:2	0.24–0.30	ovale	0.46–0.55	ovale	0.60–0.66	ovale
	1:3	0.22	ovale	0.54	ovale	0.62–0.67	ovale
	1:9	0.27–0.38	ovale	0.50–0.52	ovale	0.60–0.80	ronde
DIBK–AcOH–H_2O	8:5:1	0	—	0	—	0.05–0.10	ronde

* $CHCl_3$ = chloroforme; AcOH = acide acétique glacial; BuOH = butanol; MeOH = méthanol; EtOH = éthanol; DIBK = diisobutyl-cétone.

qu'on a pu démontrer que nos préparations de solanine possédaient un degré de pureté sinon supérieur, du moins égal à ceux des échantillons obtenus des Laboratoires Lyons de Londres et du Dr. A. ZITNAK de Guelph (Fig. 2). Cette méthode nous a aussi permis de constater que la chaconine, différemment de la solanine, est beaucoup plus soluble dans l'éthanol à 80 %.

Si nous comparons cette technique à la chromatographie sur papier, nous constatons qu'en plus des avantages inhérents à la technique elle-même, tels que rapidité, économie d'espace, de matériel, de solvant, facilité de reproduction par photographie ou décalque, usage des acides forts, etc., la chromatographie sur couches minces nous a permis de séparer les trois alcaloïdes (Fig. 2), alors qu'on ne pouvait séparer que la chaconine et la solanine par la chromatographie sur papier. De plus, après l'arrosage au chlorure d'antimoine, le papier devient raide et cassant, ce qui le rend plus difficile à manier et à conserver. Les plaques de verre n'offrent pas cet inconvénient.

Elution quantitative

Nous avons essayé d'éluer les alcaloïdes des plaques après avoir prélevé les taches avec une petite spatule et en utilisant divers solvants tels que les acides sulfurique à 1 %, chlorhydrique à 8 % ou acétique à 5 %, et l'éthanol à 95 %. Aucun de ces solvants, soit seul ou en mélange, n'a pu extraire plus de 40 à 50 % des alcaloïdes. Nous avons aussi tenté d'éluer les alcaloïdes après les avoir mélangés avec du silica gel dans une éprouvette et en nous servant des mêmes solvants. Ces essais n'ont pas donné de meilleurs résultats. La substance éluée forme un précipité avec le réactif de Marquis, ce qui empêche sa détermination par colorimétrie. Cependant, la solanidine mélangée au silica gel est extraite à 100 % avec de l'éther, mais éluée des chromatogrammes à 50 % seulement avec ce solvant. Le silica gel adsorberait donc très fortement les alcaloïdes.

Point de fusion des alcaloïdes

Le point de fusion de ces alcaloïdes purifiés fut déterminé à plusieurs reprises et comparé à ceux d'échantillons obtenus des Laboratoires Lyons de Londres et du

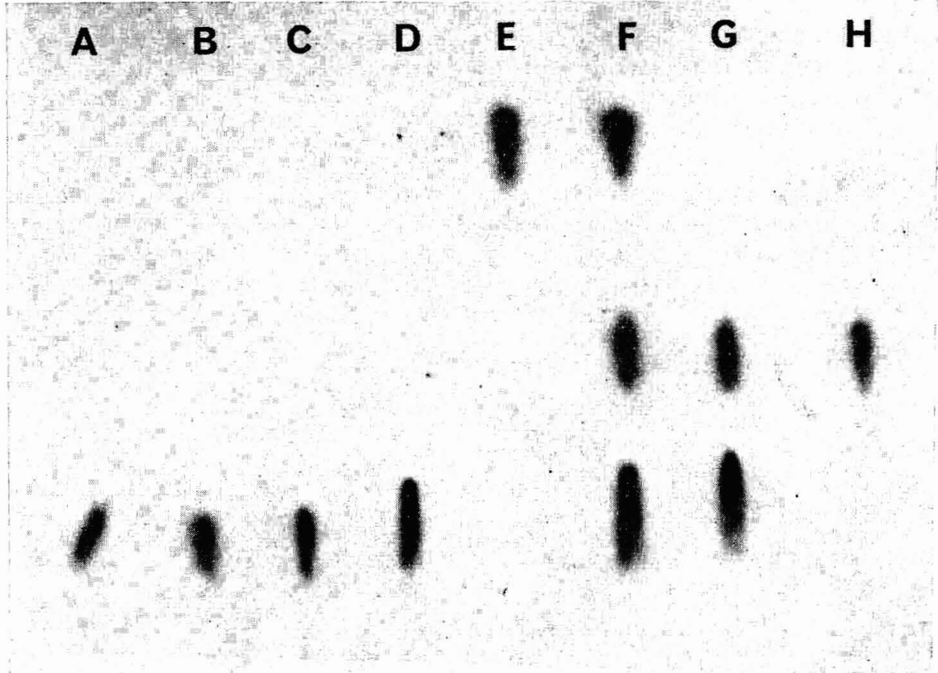

Fig. 2. Chromatographie sur couches minces de la solanine, de la chaconine et de la solanidine. Taches de gauche à droite: A: α-solanine du Dr. A. ZITNAK; B et C: deux de nos extraits d'α-solanine; D: solanine des Laboratoires Lyons de Londres; E: solanidine des Laboratoires Lyons; F: solanine, α-chaconine et solanidine; G: solanine et α-chaconine, H: α-chaconine.

Dr. A. ZITNAK de Guelph. On a constaté que ce critère de pureté qui a été utilisé très souvent dans le passé[2,8,13,14], se montrait très peu utile dans le cas de la solanine et de la chaconine. Les crystaux de solanine commencent à brunir vers 238–240°, se rétrécissent et deviennent vitreux vers 248–249°. Ils forment une masse gélatineuse vers 250–255° qui épaissit et devient noirâtre vers 260–262° en dégageant une odeur de sucre brûlé. Par contre la solanidine qui ne contient pas de sucre dans sa molécule fond rapidement à 214–216°. La chromatographie sur couches minces devient donc un moyen très recommandable de vérifier de façon rapide et pratique le degré de pureté de la chaconine et de la solanine surtout dans l'obligation où nous sommes d'apporter peu d'attention au point de fusion.

REMERCIEMENTS

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RÉSUMÉ

Une méthode rapide de séparation par chromatographie sur couches minces de la

solanine, de la chaconine et de la solanidine, est décrite dans le présent article. Elle se compare favorablement à la chromatographie sur papier pour la séparation de ces trois alcaloïdes de la pomme de terre. La détermination du point de fusion des alcaloïdes y est aussi discutée.

SUMMARY

A rapid method for the separation of the potato alkaloids, solanine, chaconine and solanidine by thin-layer chromatography is described. The results compare favourably with those obtained by paper chromatography of these compounds. The melting point determination of these alkaloids is also discussed.

BIBLIOGRAPHIE

[1] G. Ducet et S. Sémère, *Ann. Agron., Annexe II*, (1957) 141.
[2] T. A. Henry, *The Plant Alkaloids*, J & A. Churchill Ltd., Londres, 1949, p. 804.
[3] A. Zitnak, *Can. J. Biochem. Physiol.*, 39 (1961) 1257.
[4] R. Kuhn et J. Löw, *Chem. Ber.*, 88 (1955) 289.
[5] R. Kuhn et J. Löw, *Ann. Acad. Sci. Fennicae, Ser. A II*, 60 (1955) 488.
[6] V. A. Paseshnichenko et A. R. Guseva, *Biokhimiya*, 21 (1956) 606.
[7] L. C. Baker, L. H. Lampitt et O. B. Meredith, *J. Sci. Food Agr.*, 4 (1955) 197.
[8] B. E. Kline, H. von Elbe, N. A. Dahle et S. M. Kupchan, *Proc. Soc. Exptl. Biol. Med.*, 107 (1961) 807.
[9] Brinkmann Instruments Inc., *Tech. Bull.*, 20 (1962) 8.
[10] E. Pfankuch, *Biochem. Z.*, 295 (1937) 44.
[11] H. S. Rooke, J. H. Bushill, L. H. Lampitt et E. M. Jackson, *J. Soc. Chem. Ind.* (*London*), 62 (1943) 20.
[12] R. J. Block, E. L. Durrum et G. Zweig, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New-York, 1958, p. 710.
[13] D. H. Dabbs et R. J. Hilton, *Can. J. Technol.*, 31 (1953) 213.
[14] V. Prelog et O. Jeger, dans R. H. F. Manske et H. O. Holmes, *The Alkaloids, Chemistry and Physiology*, Vol. III, Academic Press, New-York, 1953, p. 247.

SOLANUM-ALKALOIDE

XVIII. DÜNNSCHICHTCHROMATOGRAPHIE VON *SOLANUM*-STEROIDALKALOIDEN UND STEROIDSAPOGENINEN*

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Im Zuge unserer Arbeiten über die Steroidalkaloidglykoside in Pflanzen der Gattungen *Solanum* und *Lycopersicon* (*Solanaceae*)[1] erwies es sich als notwendig, die Aglyka dieser Verbindungen sowie häufig gleichzeitig vorkommende Steroidsapogenine im Mikromasstab voneinander zu trennen und zu identifizieren. Hierfür erschien die Dünnschichtchromatographie besonders geeignet, eine Methode, die in den letzten Jahren auch auf dem Steroidgebiet mit grossem Erfolg angewendet wurde[2]. In einer kürzlich erschienenen Arbeit berichteten BENNETT UND HEFTMANN[3] über das dünnschichtchromatographische Verhalten einer Anzahl von Steroidsapogeninen (Spirostanolen). Die im allgemeinen stärker polaren *Solanum*-Steroidalkaloide sind bisher nur vereinzelt dünnschichtchromatographisch untersucht worden[4,5]; eine vergleichende systematische Bearbeitung steht noch aus.

Im folgenden sollen die mit 7 natürlich vorkommenden *Solanum*-Steroidalkaloiden (Aglyka) und 4 ihrer Derivate sowie mit 10 Steroidsapogeninen** erzielten Ergebnisse mitgeteilt werden. Zwei weitere Steroidalkaloide (Jervin und Conessin) sowie einige in *Solanum*-Arten nachgewiesene Phytosterine und Triterpene wurden in die Untersuchungen mit einbezogen. Auch *Solanum*-Alkaloidglykoside lassen sich dünnschichtchromatographisch gut trennen, worüber an anderer Stelle ausführlich berichtet werden soll (vgl. Zit. 5).

METHODIK

Die Chromatographie erfolgt aufsteigend bei 20° auf Platten der Grösse 13 × 25 cm. Die Entwicklungskammer kleidete man mit Fliesspapier aus, das mit dem jeweils verwendeten Lösungsmittelgemisch getränkt wurde. Die Steroide werden in Mengen von 10–30 µg in Äthanol gelöst 1.5 cm vom unteren Rand und mindestens 1.5 cm von den Seitenrändern entfernt aufgetragen. Die Steighöhe beträgt 15 cm und wird durch eine vor Beginn der Entwicklung in die Schicht eingeritzte Trennlinie begrenzt (Laufzeit mit den Entwicklungsgemischen A–C und E etwa 30–45 Min.). Die Trennung von Solanidin und Demissidin auf mit Silbernitrat imprägnierten Kieselgelschichten (Entwicklungsgemisch D) erfolgt mit Hilfe der Durchlauftechnik nach BRENNER UND NIEDERWIESER[6] (modifiziert, Laufzeit etwa 16 Stunden).

* XVII. Mitteilung: K. SCHREIBER UND H. RÖNSCH, *Experientia*, 17 (1961) 491.

** Herrn Dr. R. K. CALLOW, London, danken wir auch an dieser Stelle bestens für die freundliche Überlassung zahlreicher Steroidsapogenine.

Herstellung der Adsorptionsschichten

Die nach den folgenden Vorschriften hergestellten Suspensionen werden auf die Platten aufgegossen und durch Streichen mit einem Glasstab und Neigen der Platten gleichmässig verteilt. Die Verwendung eines Streichgeräts erwies sich als nicht notwendig.

(I) 6 g Kieselgel G nach STAHL für die Dünnschichtchromatographie (Merck) werden mit 16 ml Wasser angerührt und die gesamte Menge auf einer Platte verteilt; sie kann nach zwei Tagen lufttrocken verwendet werden (ca. 20 mg Adsorbens/cm^2).

(II) Die Platten werden nach der gleichen Vorschrift beschichtet und lufttrocken mit einer 10-% Silbernitrat-Lösung besprüht, bis die Schicht gleichmässig transparent erscheint. Die Trocknung erfolgt anschliessend bei 120° (30 Min.).

(III) Kieselsäure, wasserfrei, rein (Riedel-de Haën) wird mit 10% Gips ($CaSO_4 \cdot 0.5\ H_2O$) gemischt und durch ein Sieb von 0.25 mm Maschenweite gegeben. 25 g des Gemisches rührt man mit 43 ml Wasser an und verwendet davon 16 ml für eine Platte; es wird an der Luft getrocknet (ca. 25 mg Adsorbens/cm^2).

(IV) Aluminiumoxid, standard., zur chromatographischen Adsorptionsanalyse nach BROCKMANN (Merck), wird unter Zusatz von 10% Gips in einer Kugelmühle gemahlen und anschliessend durch ein Sieb von 0.25 mm Maschenweite gegeben. Hiervon werden 15 g mit 20 ml Wasser gemischt und auf einer Glasplatte verteilt. Die Platten können nach zweistündigem Trocknen bei 120° und Erkaltenlassen über Calciumchlorid verwendet werden (45 mg Adsorbens/cm^2).

Entwicklungsgemische

(A) Cyclohexan–Essigsäureäthylester (1:1) (alle Angaben in v/v)
(B) Chloroform–Methanol (6:4)
(C) *n*-Hexan–Triäthylamin (15:1)
(D) Essigsäureäthylester–Cyclohexan–96%-Äthanol (50:40:5)
(E) Benzin (Sdp. 80–90°)–Benzol–Essigsäureäthylester (85:5:10).

Nachweisreagenzien

Cer(IV)-sulfat–Schwefelsäure. Gesättigte Lösung von Cer(IV)-sulfat in 65% Schwefelsäure. Nach dem Besprühen wird 15 Min. auf 120° erhitzt. Das Reagens lässt sich nicht auf Al_2O_3-Schichten und auf mit $AgNO_3$ imprägnierten Schichten anwenden.

Jod. Eine Lösung von 5 g Jod und 20 g Kaliumjodid in 100 ml Wasser wird vor Gebrauch 1:50 mit Wasser verdünnt. Eine gesättigte Lösung von Jod in *n*-Hexan ist gleichfalls geeignet. Mit Silbernitrat imprägnierte Schichten müssen vor der Jod-Detektion mit gesättigter Kaliumbromid- bzw. Kaliumjodid-Lösung besprüht und anschliessend bei Raumtemperatur getrocknet werden.

Paraformaldehyd–Phosphorsäure[7]. 30 mg Paraformaldehyd werden in 100 ml konz. Phosphorsäure (D = 1.7) durch Schütteln bei Raumtemperatur gelöst. Das Reagens ist einige Wochen haltbar.

2,4-Dinitrophenylhydrazin. 0.4% Lösung in 2 *N* HCl.

Dehydrierung mit SARETT-*Reagens*[8]

40 μl wasserfreies Pyridin werden mit 4 mg fein gepulvertem Chrom(VI)-oxid versetzt, wobei sich der Pyridin–Chromsäure-Komplex als gelber Niederschlag abscheidet. Zu dieser Suspension gibt man 5 mg der zu dehydrierenden Substanz in

50 μl wasserfreiem Pyridin und lässt 12 Stunden bei Raumtemperatur stehen. Zur Chromatographie werden 10 μl des Reaktionsgemisches auf den Startpunkt aufgetragen.

ERGEBNISSE UND DISKUSSION

Für eine Gruppentrennung der untersuchten Steroide eignet sich besonders die Kombination von Adsorbens I mit Entwicklungsgemisch A. In Fig. 1 (A I) ist die relative Lage der getrennten Substanzen bzw. Substanzgruppen wiedergegeben; auf die Angabe von R_F- oder $R_{Standard}$-Werten wurde verzichtet, da diese in gewissem Umfange variieren können. In der Legende zur Abbildung sind neben den Trivialnamen die systematischen Bezeichnungen der verwendeten Spirostan-, Spirosolan- und Solanidan-Derivate sowie der weiterhin geprüften Verbindungen angeführt. Im einzelnen lassen sich mit Kombination A I folgende Trennungen erzielen: Die

Spirostan *Spirosolan*

Solanidan *Solanocapsin*

stärker polaren Verbindungen Solanocapsin (vgl. Fig. 1, Nr. 7), Jervin (Nr. 10), Conessin (Nr. 9) und Digitogenin (Nr. 20) bleiben am Startpunkt zurück. Dann folgen, nach ansteigenden R_F-Werten geordnet, die Δ^5-ungesätt.–5α-gesätt. Alkaloidpaare Solasodin–Soladulcidin (Nr. 1, 2), Tomatidenol–Tomatidin (Nr. 3, 4) und Solanidin–Demissidin (Nr. 5, 6). Auch Solasodin (Nr. 1) und sein Dehydratisierungsprodukt Solasodien (Nr. 8) lassen sich gut voneinander trennen. Die Monohydroxyspirostan-Derivate zeigen noch höhere R_F-Werte; sie lassen sich in zwei Gruppen auftrennen, und zwar in die Gruppe der Δ^5-ungesättigten (Nr. 11, 13) und 5α-gesättigten (Nr. 12, 14) Verbindungen einerseits und in die der 5β-gesättigten (Nr. 15, 16) andererseits. Das 3β-Hydroxy-12-keto-5α-spirostan-Paar Hecogenin (Nr. 17) und Sisalagenin (Nr. 18) liegt wenig oberhalb von Solanidin–Demissidin. 9-Dehydrohecogenin (Nr. 19) hat einen höheren R_F-Wert als Hecogenin (Nr. 17).

Im Chromatogramm eines von uns untersuchten Digitogenin-Präparates, dargestellt durch Hydrolyse von Digitonin (Riedel de Haën), liessen sich neben Digitogenin (Nr. 20) zwei weitere Flecken mit höheren R_F-Werten nachweisen. Bei dem in grösseren Mengen auftretenden, im Chromatogramm wenig unterhalb von Tigogenin liegenden Nebenprodukt (Nr. 22) dürfte es sich um Digalogenin handeln,

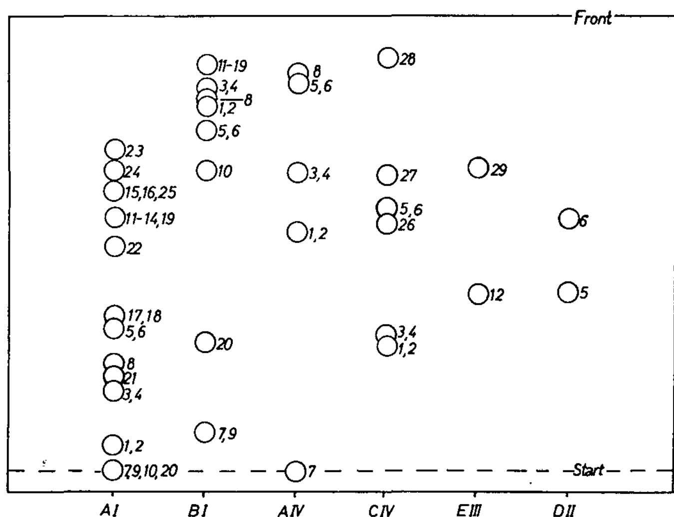

Fig. 1. Trennung von *Solanum*-Steroidalkaloiden und Steroidsapogeninen. Die römischen Zahlen bezeichnen die Adsorbentien, die grossen Buchstaben die Entwicklungsgemische. 1 = Solasodin [(22*R*:25*R*)-Spirosol-5-en-3β-ol]. 2 = Soladulcidin [(22*R*:25*R*)-5α-Spirosolan-3β-ol]. 3 = Tomatidenol [(22*S*:25*S*)-Spirosol-5-en-3β-ol]. 4 = Tomatidin [(22*S*:25*S*)-5α-Spirosolan-3β-ol]. 5 = Solanidin [Solanid-5-en-3β-ol]. 6 = Demissidin [5α-Solanidan-3β-ol]. 7 = Solanocapsin [(22*R*:23*S*:25*R*)-3β-Amino-22,26-imino-16β,23-oxido-5α-cholestan-23-ol]. 8 = Solasodien [(22*R*:25*R*)-Spirosola-3,5-dien]. 9 = Conessin [(20*S*)-3β-Dimethylamino-18,20-methylimino-pregn-5-en]. 10 = Jervin [(22*R*:23*S*:25*S*)-22,26-Imino-17α,23-oxido-14(13 → 12)abeo-cholesta-5,12-dien-3β-ol-11-on]. 11 = Diosgenin [(22*R*:25*R*)-Spirost-5-en-3β-ol]. 12 = Tigogenin [(22*R*:25*R*)-5α-Spirostan-3β-ol]. 13 = Yamogenin [(22*R*:25*S*)-Spirost-5-en-3β-ol]. 14 = Neotigogenin [(22*R*:25*S*)-5α-Spirostan-3β-ol]. 15 = Smilagenin [(22*R*:25*R*)-5β-Spirostan-3β-ol]. 16 = Sarsasapogenin [(22*R*:25*S*)-5β-Spirostan-3β-ol]. 17 = Hecogenin [(22*R*:25*R*)-12-Keto-5α-spirostan-3β-ol]. 18 = Sisalagenin [(22*R*:25*S*)-12-Keto-5α-spirostan-3β-ol]. 19 = 9-Dehydro-hecogenin [(22*R*:25*R*)-12-Keto-5α-spirost-9(11)-en-3β-ol]. 20 = Digitogenin [(22*R*:25*R*)-5α-Spirostan-2α,3β,15β-triol]. 21 = Gitogenin (?)[(22*R*:25*R*)-5α-Spirostan-2α,3β-diol]. 22 = Digalogenin (?) [(22*R*:25*R*)-5α-Spirostan-3β, 15β-diol]. 23 = Cycloartenol [9β,19-Cyclo-5α-lanost-24-en-3β-ol]. 24 = 4α-Methyl-5α-stigmasta-7, 24(28)-dien-3β-ol. 25 = β-Sitosterin [Stigmast-5-en-3β-ol]. 26 = 5α-Solasodan-3-on [(22*R*:25*R*)-5α-Spirosolan-3-on]. 27 = 5α-Tomatidan-3-on [(22*S*:25*S*)-5α-Spirosolan-3-on. 28 = 5α-Solanidan-3-on. 29 = Tigogenon [(22*R*:25*R*)-5α-Spirostan-3-on].

das von TSCHESCHE und Mitarb.[9] u.a. auch aus Handelsdigitonin isoliert und in seiner Konstitution aufgeklärt wurde. Dieser Befund liess sich durch das Molekül-Massenspektogramm[10] des Präparats bestätigen, in dem 16 Masseneinheiten unterhalb der Digitogenin-Linie eine weitere Linie auftrat. Die zweite Begleitsubstanz (Nr. 21), vermutlich Gitogenin[9], hat einen wesentlich niedrigeren R_F-Wert und liess sich nur in Spuren nachweisen.

Am weitesten laufen die mitchromatographierten Sterine und Triterpene, und zwar β-Sitosterin (Nr. 25), 4α-Methyl-5α-stigmasta-7,24(28)-dien-3β-ol (Nr. 24) und Cycloartenol (Nr. 23), die gleichfalls in *Solanum*-Arten vorkommen können[11]*.

Die mit Kombination A I am Startpunkt verbleibenden Verbindungen Solanocapsin, Conessin, Jervin und Digitogenin können mit Entwicklungsgemisch B chromatographiert werden (vgl. Fig. 1, B I). Die beiden erstgenannten Steroidalkaloide laufen auch hier zusammen und haben den niedrigsten R_F-Wert; ihre Trennung

* Cholesterin, 5α-Cholestan-3β-ol, Campesterin und Stigmasterin zeigen ein gleiches chromatographisches Verhalten wie Nr. 25, Lophenol wie Nr. 24 und das Triterpen Parkeol wie Nr. 23.

wurde nicht weiter versucht, da ein gemeinsames Vorkommen unwahrscheinlich ist. Jervin und Digitogenin liessen sich gut differenzieren. Die weiterhin geprüften Steroide befinden sich nahe der Lösungsmittelfront und werden nicht mehr gut getrennt. Bemerkenswert ist, dass Solanidin und Demissidin mit Entwicklungsgemisch B eine kürzere Laufstrecke haben als die Spirosolan-Alkaloide, während sie mit System A weiter wandern.

Eine etwa gleich gute Trennung der angeführten Steroide ist auch auf Aluminiumoxidschichten möglich, was am Beispiel der *Solanum*-Steroidalkaloide gezeigt wird (vgl. Fig. 1, C IV).

Für den Nachweis der Substanzen sind mehrere Sprühreagenzien brauchbar. Mit Cer(IV)-sulfat–Schwefelsäure lassen sich auf Kieselgelschichten alle untersuchten Steroide sichtbar machen: Nach dem Erhitzen bräunlich- bis schwarzgraue Flecken, die unter der U.V.-Lampe fluoreszieren. Dabei zeigen Hecogenin und Sisalagenin eine leuchtend hell-blaue Fluoreszenz, das vermutliche Digalogenin fluoresziert gelblich und alle anderen rötlich-violett bis weiss-violett (Empfindlichkeit etwa 0.1 μg). Die Δ^5-ungesättigten Verbindungen reagieren bereits in der Kälte. Die Alkaloide lassen sich vorteilhaft mit Jod nachweisen, wobei gelbe bis braune Färbungen auftreten (Nachweisgrenze ebenfalls etwa 0.1 μg). Steroidsapogenine, Sterine und Triterpene reagieren mit diesem Reagens weniger empfindlich, so dass hiermit eine weitere Differenzierungsmöglichkeit für basische und neutrale Steroide gegeben ist. Darüber hinaus besitzt Jod als indifferentes Nachweisreagens für die präparative Dünnschichtchromatographie grosse Vorteile[12]: die hiermit erhaltenen Färbungen verblassen relativ schnell, und die Substanzen lassen sich durch Elution der markierten Zonen unverändert zurückgewinnen.

Wie die Chromatographie mit den Kombinationen A I, B I und A IV zeigt, lassen sich die Δ^5-ungesätt.–5α-gesätt. Steroidpaare, und zwar sowohl bei den Solanidanen und Spirosolanen als auch bei den Spirostanen, nicht auftrennen. Für den selektiven Nachweis von 3β-Hydroxy-Δ^5-Verbindungen eignet sich Paraformaldehyd–Phosphorsäure. Etwa 10 Min. nach dem Besprühen erscheinen rotviolette (Alkaloide) bzw. blaue (Sapogenine) Flecken. Solasodien gibt eine Rotfärbung. Auch 3β-Hydroxy-5α-Verbindungen lassen sich im Gemisch mit den entsprechenden Δ^5-ungesättigten selektiv nachweisen, wenn sie vor der Chromatographie in die jeweiligen 3-Keto-Derivate überführt werden. Dies gelingt durch schonende Dehydrierung mit SARETT-Reagens[8], das die 3β-Hydroxy-Δ^5-Steroide nicht angreift. Die Reaktion verläuft unter den gegebenen Bedingungen nicht ganz quantitativ; es wird jedoch soviel Keton gebildet, dass ein Nachweis mit 2,4-Dinitrophenylhydrazin keine Schwierigkeiten bereitet. Die Chromatographie führt man bei den Alkaloidpaaren (3β-Hydroxy-Δ^5–3-Keto-5α-Verbindung) auf Adsorbens IV mit Entwicklungsgemisch C durch, bei Diosgenin–Tigogenon auf Adsorbens III mit System E (vgl. Fig. 1). Die Ketone haben stets die höheren R_F-Werte. Die Δ^5-ungesättigten Verbindungen weist man auf dem unteren Teil der Platte mit Paraformaldehyd–Phosphorsäure nach, die aus den 5α-gesättigten Steroiden entstandenen Ketone auf der oberen Hälfte des Chromatogramms mit 2,4-Dinitrophenylhydrazin.

Eine Trennung von Solanidin und Demissidin, jedoch nicht von Solasodin und Soladulcidin bzw. Tomatidenol und Tomatidin, gelingt auch auf mit Silbernitrat imprägnierten Kieselgelschichten mit dem Entwicklungsgemisch D (vgl. Fig. 1, D II). Hierbei wird die Komplexbildungstendenz zwischen Doppelbindungen und Silber-

ionen ausgenutzt, eine Methode, die kürzlich zur chromatographischen Trennung auch anderer Stoffgruppen mit Erfolg angewendet worden ist[13].

Zusammenfassend kann somit festgestellt werden, dass sich sowohl natürlich vorkommende *Solanum*-Steroidalkaloide (Aglyka) als auch gleichzeitig anwesende Steroidsapogenine durch einfache adsorptive Dünnschichtchromatographie an Kieselgel gruppenweise gut trennen lassen. Hierbei erfolgt bei den Spirosolan-Alkaloiden im Gegensatz zu den Spirostan-Sapogeninen auch eine Differenzierung der an C-25 isomeren Verbindungen. Im Unterschied zu den natürlichen Sapogeninen, die ohne Ausnahme 22*R*-Konfiguration besitzen, ist bei den Spirosolan-Alkaloiden Konfigurationsänderung an C-25 mit gleichzeitigem Wechsel der Konfiguration auch an C-22 verbunden, so dass vermutlich die letztere für das unterschiedliche dünnschichtchromatographische Verhalten der Alkaloide verantwortlich ist. Eine Differenzierung der Δ^5-ungesätt.–5α-gesätt. 3β-Hydroxy-Steroidpaare lässt sich auf indirektem Wege erreichen, wenn die gesättigten Verbindungen vor der Chromatographie selektiv zu den entsprechenden 3-Ketonen dehydriert werden. Solanidin und Demissidin sind ausserdem auf mit Silbernitrat imprägnierten Kieselgelschichten trennbar.

ZUSAMMENFASSUNG

Die Anwendung der Dünnschichtchromatographie für die Trennung von sieben *Solanum*-Steroidalkaloiden (Solanidin, Demissidin, Tomatidenol, Tomatidin, Solasodin, Soladulcidin, Solanocapsin), vier ihrer Derivate (5α-Solanidan-3-on, 5α-Tomatidan-3-on, 5α-Solasodan-3-on, Solasodien) sowie von zehn Steroidsapogeninen wird beschrieben. Zwei weitere Steroidalkaloide (Jervin, Conessin) und einige Phytosterine und Triterpene wurden in die Untersuchungen einbezogen. Die genannten Steroide lassen sich durch einfache adsorptive Dünnschichtchromatographie an Kieselgel gruppenweise trennen. Möglichkeiten einer weiteren Differenzierung dieser Gruppen werden mitgeteilt.

SUMMARY

The application of thin-layer chromatography to the separation of mixtures of seven steroidal *Solanum* alkaloids (solanidine, demissidine, tomatidenol, tomatidine, solasodine, soladulcidine, solanocapsine), four of their derivatives (5α-solanidan-3 one, 5α-tomatidan-3-one, 5α-solasodan-3-one, solasodiene) as well as of ten steroidal sapogenins has been investigated. Two further steroidal alkaloids (jervine, conessine) and some phytosterols and triterpenes have also been studied. The above-mentioned steroids can be resolved into groups by simple adsorption chromatography on thin layers of silica gel. Some possibilities for a further differentiation of these groups are described.

LITERATUR

1 K. Schreiber, U. Hammer, E. Ithal, H. Ripperger, W. Rudolph und A. Weissenborn, *Tagungsber. Deut. Akad. Landwirtschaftswiss. Berlin*, Nr. 27 (1961) 47;
K. Schreiber, U. Hammer, U. Hof, E. Ithal und W. Rudolph, *ibid.*, Nr. 27 (1961) 75.
2 E. Stahl (Herausgeber), *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1962;
K. Randerath, *Dünnschicht-Chromatographie*, Verlag Chemie, Weinheim/Bergstrasse, 1962.
3 R. D. Bennett und E. Heftmann, *J. Chromatog.*, 9 (1962) 353.
4 G. Adam, *Dissertation Universität Jena*, 1962;

B. ANGERMANN, *Dissertation Freie Universität Berlin*, 1960;
F. BIANCHI, *persönliche Mitteilung vom* 1.4.1961;
S. HEŘMÁNEK, V. SCHWARZ UND Z. ČEKAN, *Collection Czech. Chem. Commun.*, 26 (1961) 1669; *Pharmazie*, 16 (1961) 566;
R. KUHN UND I. LÖW, *Chem. Ber.*, 95 (1962) 1748;
H. RÖNSCH, *Dissertation Universität Jena*, 1962;
H. SANDER *et al.*, *Planta Medica*, 9 (1961) 8; *Flora*, 151 (1962) 150; *Arch. Pharm.*, 295 (1962) 6;
K. SCHREIBER *et al.*, *Experientia*, 17 (1961) 491; *Z. Chem.*, 3 (1963) 100; *Ann.*, im Druck.
[5] P. M. BOLL, *Acta Chem. Scand.*, 16 (1962) 1819;
K. SCHREIBER UND H. RÖNSCH, *Tetrahedron Letters*, Nr. 5 (1963) 329.
[6] M. BRENNER UND A. NIEDERWIESER, *Experientia*, 17 (1961) 237.
[7] E. G. C. CLARKE, *Nature*, 181 (1958) 1152; vgl. Zit. 1.
[8] G. I. POOS, G. E. ARTH, R. E. BEYLER UND L. H. SARETT, *J. Am. Chem. Soc.*, 75 (1953) 422.
[9] R. TSCHESCHE UND G. WULFF, *Chem. Ber.*, 94 (1961) 2019;
R. TSCHESCHE, G. WULFF UND G. BALLE, *Tetrahedron*, 18 (1962) 959.
[10] M. v. ARDENNE, K. STEINFELDER, R. TÜMMLER UND K. SCHREIBER, *Experientia*, 19 (1963) 178.
[11] K. SCHREIBER UND G. OSSKE, *Kulturpflanze*, 10 (1962) 372; *Experientia*, 19 (1963) 69.
[12] G. ADAM UND K. SCHREIBER, *Z. Chem.*, 3 (1963) 100.
[13] B. DE VRIES, *Chem. Ind. (London)*, (1962) 1049;
C. B. BARRETT, M. S. J. DALLAS UND F. B. PADLEY, *Chem. Ind. (London)*, (1962) 1050;
L. J. MORRIS, *Chem. Ind. (London)*, (1962) 1238;
J. HERLING, J. SHABATAI UND E. GIL-AV, *J. Chromatog.*, 8 (1962) 349.

THIN-LAYER CHROMATOGRAPHY OF ISOMERIC OXIMES. I.

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Theoretically two isomeric forms of each aldoxime and unsymmetrical ketoxime are possible. A review of the literature indicates that in the reaction of carbonyl compounds with hydroxylamine very often only one product is obtained. It is, however, doubtful whether the isolated products, although having constant melting points, always represent samples of pure isomers.

The only successful separation of isomeric oximes was achieved in the case of benzoin oximes and anisoin oximes by adsorption chromatography applying the brush method[1]. The authors, however, claim that the application of this method is somewhat limited by the catalytic action of the adsorbent on the isomerization. To our knowledge, no separation by paper chromatography has up to now been achieved. Infrared spectroscopic data[2] indicate the possibility of distinguishing the isomeric oximes on the basis of the position of the OH stretching frequency. None of these methods, however, can guarantee the presence of one single isomer in the isolated product.

In view of the lack of a method for the analytical separation and rapid determination of isomeric oximes, we have studied the thin-layer chromatography of the following oximes: α- and β-benzaldoximes, α- and β-benzoin oximes, and, α- and β-anisoin oximes.

Following the directions given in the literature[3] α- and β-benzaldoximes were obtained in the pure state. The preparation of α- and β-benzoin oximes was carried out according to WERNER AND DETSCHEFF[4]. α-Benzoin oxime was easily obtained in the pure state by crystallization from dilute alcohol, whereas the β-form showed two spots on the chromatogram. The preparation of the β-form was attempted without any heating of the reaction mixture, but the resulting product, purified as given in the literature, was shown by thin-layer chromatography to be, in spite of its correct melting point, a mixture of both isomeric forms. A chromatographically pure sample of the β-form was obtained only after recrystallization from an ether–petroleum ether mixture. The oximation of anisoin, prepared according to BÖSLER[5], was carried out as described by ZECHMEISTER[1]. A pure sample of the α-form was isolated from the reaction mixture by recrystallization from an ether–petroleum ether mixture. The pure β-form was obtained as follows: the reaction product (oximation performed without heating) was dissolved in hot benzene and the solution was frozen; when the mixture was left at room temperature crystals separated which were sparingly soluble in benzene. Successive treatment with benzene and centrifuging of the suspension gave a sample of the pure β-anisoin oxime.

Good separation with very different R_F values of isomeric oximes (Table I) was

achieved on thin layers of silica gel G (according to STAHL) using benzene–ethyl acetate and benzene–absolute methanol, respectively.

TABLE I

R_F VALUES OF OXIMES

Compound	$R_F \times 100$	
	α-Form	*β-Form*
Benzaldoxime	50	32
Benzoin oxime	14	37
Anisoin oxime	5	23

Fig. 1 shows the separation of α- and β-benzaldoximes, α- and β-benzoin oximes, and α- and β-anisoin oximes, using benzene–ethyl acetate (50:10 v/v).

The spots were detected by spraying the plate with aqueous 0.5 % cupric chloride solution, when the oximes appeared as coloured spots of oxime–metal complexes. In the case of isomeric benzoin oximes and anisoin oximes, each isomer gave a different colour. The complex of the α-isomer was green and that of the β-form brown; the latter turned to green within a short period indicating possible isomerization of the complex. The pure isomeric oximes, however, are stable on silica gel, since brown and green spots appeared on spraying plates which had been left to stand for several days after development.

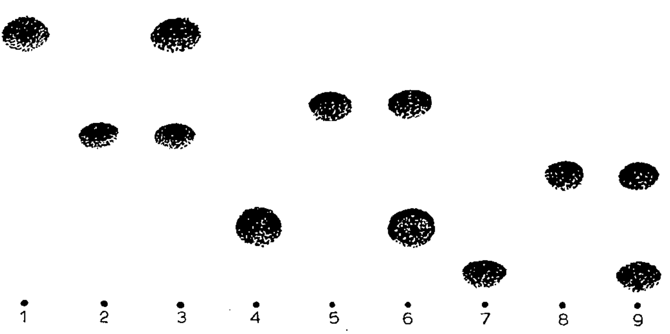

Fig. 1. Thin layer chromatogram of isomeric oximes. The oximes were dissolved in tetrahydrofuran and 10 μl of a 0.5 % (w/v) solution of each oxime was applied to the plate. Adsorbent: silica gel G (according to STAHL). Solvent system: benzene–ethyl acetate (50:10 v/v). Solvent front 14 cm. Spraying reagent: saturated alcoholic solution of cupric acetate monohydrate. (1) α-Benzaldoxime; (2) β-Benzaldoxime; (3) Mixture of isomeric benzaldoximes; (4) α-Benzoin oxime; (5) β-Benzoin oxime; (6) Mixture of isomeric benzoin oximes; (7) α-Anisoin oxime; (8) β-Anisoin oxime; (9) Mixture of isomeric anisoin oximes.

α-Benzaldoxime failed to give a colour reaction with the above reagent, but when the plate was sprayed with a saturated alcoholic solution of cupric acetate monohydrate and kept at 100° for 10 min, the green colour of the complex was easily obtained.

It has been observed that the isomeric oximes, when kept in solutions of tetrahydrofuran or alcohol for a period of two to three days exhibited a marked tendency to isomerization.

The separation of isomeric oximes was also performed on a micro preparative scale.

Thin-layer chromatography has proved a convenient and rapid method for analytical separation and determination of isomeric oximes.

EXPERIMENTAL

Materials

α-Benzaldoxime, m.p. 35°; β-benzaldoxime, m.p. 130°; α-benzoin oxime, m.p. 151°; β-benzoin oxime, m.p. 99°; α-anisoin oxime, m.p. 123°; β-anisoin oxime, m.p. 125.5°. Melting points are not corrected.

Solvent systems

Benzene–ethyl acetate (50:10 v/v); benzene–absolute methanol (50:10 v/v).

Spraying reagents

Aqueous 0.5 % cupric chloride solution; saturated alcoholic cupric acetate monohydrate solution; alcoholic ferric chloride solution.

Method

Smooth glass plates (20 × 13 × 0.5 cm) were coated with a 0.2 mm thick layer of standardized silica gel G (according to STAHL) by means of a home-made applicator. The plates were then dried in an oven at 110° for 10 min, allowed to cool and kept in a moisture-free chamber. Spots of 0.5 % solutions of oximes in tetrahydrofuran (w/v) were applied from a capillary pipette in a line 2.5 cm from one edge of the plate, at intervals of 2 cm. The development was carried out by the ascending method in closed rectangular tanks (24 × 16 × 8 cm), saturated for 1 h with the appropriate solvent system. The tanks were lined with filter paper for good equilibration. The time required for the development (the solvent front moved 14 cm) was approximately 45 min. The developed chromatograms were then removed from the tanks, dried and sprayed with the detecting reagent.

For micro preparations the solutions were applied as bands of continuous spots, and the plates were developed twice. A narrow strip of the adsorbent was then sprayed and used as a guide for scraping the bands from the glass surface. The silica gel portions containing single isomers were extracted, the extracts evaporated at room temperature and the solids obtained applied separately on a new plate. Each extract gave only one spot.

SUMMARY

α- and β-Benzaldoximes, α- and β-benzoin oximes, and α- and β-anisoin oximes have been separated by thin-layer chromatography. In view of the highly different R_F values and the simple procedure, this method can be used for analytical separation, rapid determination and semimicro preparation of isomeric oximes.

REFERENCES

[1] L. ZECHMEISTER, W. H. MCNEELY AND C. SOLYOM, *J. Am. Chem. Soc.*, 64 (1942) 1923.
[2] A. PALM AND H. WERBIN, *Can. J. Chem.*, 31 (1953) 100.
[3] A. I. VOGEL, *A Textbook of Practical Organic Chemistry*, Longmans, Green & Co., London, 1948, p. 683.
[4] A. WERNER AND TH. DETSCHEFF, *Ber.*, 38 (1905) 69.
[5] M. BÖSLER, *Ber.*, 14 (1881) 323;
R. STIERLIN, *Ber.*, 22 (1889) 376.
[6] W. HIEBER AND F. LEUTERT, *Ber.*, 60 (1927) 2308.

THE SEPARATION OF THE RARE EARTHS BY PARTITION CHROMATOGRAPHY WITH REVERSED PHASES

PART I. BEHAVIOUR OF COLUMN MATERIAL

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The great similarities in the behaviour of the rare earth elements in aqueous solution have resulted in the separation of individual members being difficult to accomplish, and properties which can provide the basis of a method for the separation of the elements, usually differ so slightly through the series, that any fractionation process must be repeated many times to achieve isolation of a single lanthanide.

Thus early fractional crystallisation procedures proved long and tedious when valency differences could not be used[1], and it was only when the different distributions of the rare earths in solid cation-exchanger–aqueous complexing agent systems were exploited by chromatographic techniques, that practical separations were achieved[2]. Recently, however, it has been shown that certain liquid extractants can provide higher separation factors for the rare earths than have so far been obtained in cation-exchange systems[3], but again multiple equilibrations are required to isolate individual elements in the same valency state, and the difficulty of conveniently carrying out multiple small-scale liquid–liquid extractions has precluded their widespread application.

It would therefore appear that rare earth separations might be considerably simplified if liquid extractants could be incorporated into a scheme of chromatographic separation and thus combine the selectivity built into a suitable complexing agent with the technical advantages of chromatographic operation. The possibility of retaining a complexing agent on a solid supporting medium has already been considered[4–9], and columns of this type of material used for the separation of elements prior to analytical determination[10–13]. Clearly rare earth separations might be devised using this type of reversed phase partition technique, if a suitable complexing agent could be held sufficiently firmly on a solid support, and preliminary investigations indicated that the alkyl-phosphoric acid, di-(2-ethylhexyl) orthophosphoric acid (HDEHP), which has been shown to give the good average separation factor of 2.5 for adjacent rare earths[14], could be conveniently retained on a poly-(vinyl chloride/vinyl acetate) copolymer, (Corvic). Further, reaction between metal and ligand occurs rapidly, an essential requirement for elution peaks to be sharp. Reversed phase partition chromatography might also be expected to possess the advantages over conventional liquid–liquid extraction of reduced emulsification or physical entrainment of the extractant, reduced spill hazard, and more convenient operation in

restricted spaces (*e.g.* gloveboxes). In a short preliminary communication[15] the separation of all the rare earths by chromatographic elution from HDEHP–Corvic columns has been reported. In this paper some of the investigations carried out to assess the behaviour of the column material are described more fully.

EXPERIMENTAL

Column material

The poly-(vinyl chloride/vinyl acetate) copolymer, (Corvic, Imperial Chemical Industries Ltd.), used as solid support material was the commercially available product sieved to obtain particles of a suitable size (100–150 mesh).

HDEHP was obtained from two different commercial manufacturers and purified by a method based on that described elsewhere[16]. It was found that the distribution ratios for the extraction of a number of lanthanides from a perchloric acid phase into toluene solutions of the two different samples of HDEHP, after purification, matched within the limits of experimental error.

Column material was prepared by dissolving the requisite volume of HDEHP in a mixture of 3 ml of redistilled toluene and 5 ml of chloroform, and the resulting solution added to 10 g of the copolymer. Surplus organic solvent was evaporated off in a current of air and when constant weight had been achieved the powder containing the HDEHP stored for use. When required it was slurried with an aqueous phase and made up into a column in the usual way. Unless otherwise stated it will be assumed in this text that column material has been prepared by adding 2 ml of purified HDEHP to 10 g of the copolymer.

Radio tracers

Radio tracers were obtained by irradiating "Specpure" chemicals in a neutron flux of $\sim 10^{12}$ n/cm^2/sec and after irradiation the γ-spectra and decay curves of these tracers examined for interfering radiations. Interference from impurities or daughter products was avoided by careful choice of irradiation and decay times, by the use of γ-spectrometry or by chemical separations. When chemical separation was necessary the required lanthanide was eluted from an HDEHP-Corvic column with perchloric acid at 60°.

Apparatus

All glassware was Pyrex. Chromatographic tubes used to contain the columns were 120 mm long $\times$ 5 mm diameter and were fitted with water jackets fed from a thermostat, so that the running temperature of the columns could be controlled. In all work reported in this paper, columns were maintained at $60° \pm 0.5°$ since a series of preliminary experiments indicated that increasing the temperature increased the sharpness of the elution peaks. It was found that at 60° sharp elution peaks were obtained without bubble formation at the top of the column presenting any undue difficulty. Column effluents were passed through a drop counter to a fraction collector, and collected in volumes of 0.5 ml or less. The radioactivity of solutions was usually assayed by means of a 2 in. $\times$ $1^1/_2$ in. NaI (Tl) well crystal, used with a simple scaler and timing set, but where γ-ray spectra were required a 3 in. $\times$ 3 in. NaI (Tl) crystal was used in conjunction with a 99 channel pulse analyser.

Capacity determinations

In order to obtain a value for the maximum quantity of element that could be extracted from aqueous solution by HDEHP–Corvic column material, 1.000 ± 0.003 g of polymer retaining the complexing agent was made up into a column and a standard solution of a lanthanide, "spiked" with radioactive tracer, was added as influent in very dilute perchloric acid. For different experiments both lanthanium and europium were used. When the activity of the effluent reached that of the influent, the column was washed with dilute perchloric acid until it was not possible to detect any further activity leaving the column, and the sorbed metal was eluted with 8 M perchloric acid. The effluent containing the metal that had been retained by the, column was then made up to a known volume and the metal concentration of this solution calculated after comparing the count of 2 ml with that of 2 ml of the original standard solution. In a series of experiments carried out with Corvic treated with toluene and chloroform but no HDEHP it was found that the amount of metal extracted was always less than 0.02 mg of lanthanide/g of column material.

Elutions

1.000 ± 0.003 g of HDEHP–Corvic column material was slurried with $N/500$ perchloric acid and made up to form a column 5 mm diameter × 10 cm long, thermostated to 60°. Lanthanide tracer was sorbed to form a thin band at the top of the column by adding a dilute solution of the metal in $N/500$ perchloric acid as influent, and the column was washed with $N/500$ perchloric acid. An elution curve was then obtained by eluting the lanthanide with the required aqueous phase, and monitoring the effluent for activity.

RESULTS AND DISCUSSION

It is essential that any column material that is to be used for chromatography may be prepared in reproducible form, and that its behaviour does not vary over the different time intervals that may elapse between preparation and use. Whilst liquid complexing agents are probably more readily purified than a solid material containing complexing groups chemically bound to the matrix, there might well be difficulty in obtaining reproducible behaviour from a column material prepared by sorbing the reagent onto a powdered solid. In order to assess the reliability of the method described in the experimental section for making up reproducible batches of column material, over 50 elutions of lanthanum from HDEHP–Corvic columns were carried out. The peak effluent volume for similar runs was found to vary by less than 3 % from the mean value, and to be independent, within the limits of experimental error, of the time that the column material had been allowed to stand between preparation and use (up to 11 days). Elution of europium from a sample of column material prepared 64 days before use was also carried out and the peak effluent volumes obtained were the same as obtained for freshly prepared material.

Capacity of column material

In order to assess the metal extraction capacity of HDEHP–Corvic mixtures, a series of batches of the column material were made up with different HDEHP loadings. HDEHP is known to be dimeric in benzene and naphthalene but monomeric in

ethylene glycol[16]. Assuming one exchangeable hydrogen ion for each acid dimer, the cation-exchange capacity of the column material, calculated from the added HDEHP is given in Table I, together with that found from metal adsorption measurements.

It can be seen in all cases that the added HDEHP appears to be available for complex formation with the rare earths and in fact more metal is extracted than would be expected on the assumption that 1 mole of M^{3+} combines with six formula weights

TABLE I

CAPACITY OF HDEHP–CORVIC MIXTURES OF DIFFERENT LOADINGS CALCULATED FROM AMOUNT OF HDEHP ADDED (A) AND METAL EXTRACTED AT SATURATION (B)

Mixture	*Capacity of column material (mequiv./g)*		*No. of determinations*
	A	*B*	
1	0.070	0.090	5
2	0.099	0.150	4
3	0.135	0.223	7
4	0.194	0.276	5
5	0.247	0.322	16
6	0.298	0.408	4

(three dimer weights) of HDEHP. Investigations into the behaviour of HDEHP in liquid–liquid systems under metal rich conditions are severely limited by gel formation when the rare earth content of the organic phase is slightly in excess of the 1:6 metal–ligand ratio and therefore the reason for this behaviour is difficult to assess. No attempt has been made to calculate the degree of polymerisation of the HDEHP in the solid phase.

Flow rate

Reaction between HDEHP and the lanthanides is known to be rapid in liquid–liquid systems and if distribution of the elements also occurs quickly when the complexing agent is immobilised on a solid phase, efficient utilisation of the column will be favoured, resulting in sharp elution curves. The shape of the elution curves was found to improve as the running temperature of the column was increased, and 60° was eventually used as a convenient temperature at which good elutions could be achieved without any technical difficulty. Using the formula, number of theoretical plates $= 2\,s\,(s - t)/d^2$, where s = the volume of the eluate at peak maximum, t = the interstitial column volume, and d = half width of peak at $1/e$ of maximum solute concentration, the number of theoretical plates for different flow rates at 60° were calculated for the elution of lanthanides from HDEHP–Corvic columns. These results are recorded in Fig. 1. Completely constant flow rates were difficult to obtain due to the tendency of the columns to pack down slightly during flow of the liquid phase, and this gave rise to scatter of points, but it can be seen that at low flow rates the height of a theoretical plate is similar to that obtained when ion-exchangers are used for rare earth separations[17].

Elution of the elements

Distribution of a metal between an aqueous phase and a solid medium is usually most satisfactorily followed by batch extraction techniques and it has been found possible to investigate the behaviour of some complexes sorbed on solids by this method[8].

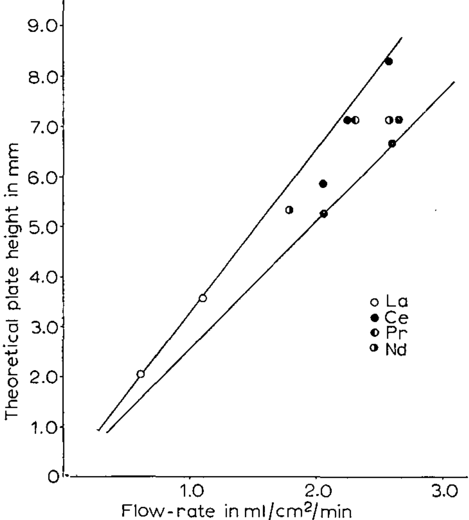

Fig. 1. Variation of plate height with flow rate.

However, after a number of preliminary extractions this technique was considered unsatisfactory for HDEHP–Corvic mixtures, since upon prolonged vigorous shaking, there was evidence that small quantities of HDEHP were washed off the more heavily loaded supports, consequently all distribution data were obtained from elution curves.

Equations have been derived for the chromatographic elution of elements from papers impregnated with ion-exchangers[18], and from phases consisting of complexing agents retained on columns[19] and paper strips[20]. In all cases the reaction between metal and column material is given by the equation:

$$M^{n+} + n\ HL_0 \rightleftharpoons ML_{n0} + n\ H^+$$

where the subscript o refers to species present in the solid phase. All these equations reduce to the form:

$$R_M = \log A_s/A_m + \log K^{11} + n \log [HL]_0 - n \log [H^+] \quad (1)$$

where the zone migration parameter $R_M = (1/R_F - 1)$ and A_s and A_m are the cross-sectional areas of the stationary and mobile phases respectively. Under conditions of constant ionic strength concentrations are often used in place of activities when the constant K^{11} then refers to a standard set of conditions.

Consequently it can be seen that if the column parameter A_s/A_m, the constant K^{11} and the HDEHP loading of the column do not vary there should be a linear

dependency of R_M upon the log of the hydrogen ion concentration of the aqueous elutrient, the slope being — n. Further, at constant acidity there should be an n'th power dependency of $(1/R_F - 1)$ upon $[HL]_0$, but in this case it is unlikely that concentrations may reasonably be used in place of activities. The value of n has been found to be 3 for the extraction of the lanthanides by HDEHP in liquid–liquid systems, and results obtained for the elution of europium from Corvic columns of different HDEHP loadings, which are presented on a plot of R_M *versus* log $[H^+]$ in Fig. 2, again indicate a value of 3 for n in all cases. Thus similar acid dependencies are obtained for column operation and for equilibrium liquid–liquid measurements and are independent of HDEHP loadings over the values investigated. Similar results have been reported using paper impregnated with HDEHP[20]. Whilst perchloric

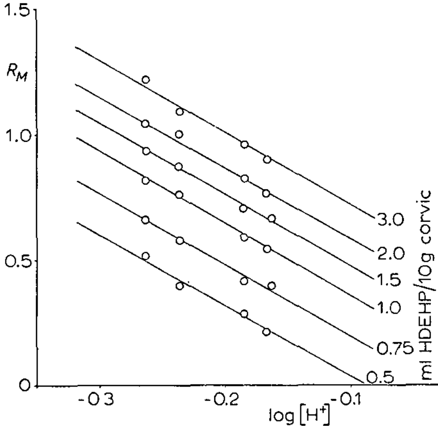

Fig. 2. Elution of europium by perchloric acid from Corvic columns retaining varying amounts of HDEHP.

acid only was used for these experiments and the ionic strength of the aqueous phase was not maintained constant, the differences that occurred were found not to affect the position of the peak maximum over quite large changes in perchlorate ion concentration. No attempt was made to relate R_M to $[HL]_0$ since the nature of the non-aqueous phase is not yet known, but from Fig. 2 it can be seen that an increase in column loading is accompanied by an increase in R_M.

CONCLUSIONS

Suitable columns for the chromatographic elution of the rare earths can be made up from HDEHP–Corvic mixtures. Batches can be prepared with reproducible elution behaviour, and the peak effluent volume of the eluted element does not appear to be dependent upon the time that is allowed to elapse between preparation and use of the column material over the periods investigated. The capacity of the columns used in these determinations is lower than that of a number of ion-exchangers but elution of the elements is by aqueous acid solutions and the height of an equivalent theoretical plate at low flow rates approaches that obtained in conventional ion-exchanger–complexing agent systems. However, the actual separation of individual

rare earths from each other will be dependent upon the good separation factors which are found when HDEHP is used for liquid extractions, prevailing when the HDEHP is sorbed on Corvic. Whilst the third power dependency of $(1/R_F - 1)$ on the hydrogen ion concentration of the eluting phase indicates close similarities in the performance of HDEHP in the liquid–liquid and liquid–solid systems, clearly for a more comprehensive comparison it is necessary to have data for the behaviour of all the rare earths in the two cases. This will be considered in the second paper in this series.

SUMMARY

The complexing agent di-(2-ethylhexyl) orthophosphoric acid, which is known to have a good average separation factor for adjacent rare earths, has been retained on a poly-(vinyl chloride/vinyl acetate) copolymer, and this mixture used as column material for chromatographic elution of the rare earths. This column material can be conveniently prepared with reproducible behaviour, sharp elution peaks being obtained at a running temperature of 60°, and a third power dependency of $(1/R_F - 1)$ upon the hydrogen ion concentration of the eluting phase is found at several different loadings of the polymer with complexing agents.

REFERENCES

[1] J. K. MARSH, *Quart. Rev. (London)*, 1 (1947) 126.
[2] J. E. POWELL, in F. H. SPEDDING AND A. H. DAANE (Editors), *The Rare Earths*, John Wiley & Sons Inc., New York, 1961.
[3] D. F. PEPPARD AND G. W. MASON, in E. V. KLEBER (Editor), *Rare Earth Research*, Macmillan, New York, 1961.
[4] D. K. HALE, *Brit. Pat.*, 738,500.
[5] J. W. WINCHESTER, *U.S.A.E.C. Documents*, O.R.N.L. 60-3-158 (1960) and 58-12-48 (1958).
[6] S. SIEKIERSKI AND I. FIDELIS, *J. Chromatog.*, 4 (1960) 60.
[7] A. G. HAMLIN AND B. J. ROBERTS, *Nature*, 185 (1960) 527.
[8] T. B. PIERCE, *Anal. Chim. Acta*, 24 (1961) 146.
[9] E. CERRAI AND C. TESTA, *Energia Nucl. (Milan)*, 8 (1961) 510.
[10] A. G. HAMLIN, B. J. ROBERTS, W. LAUGHLIN AND S. G. WALKER, *Anal. Chem.*, 33 (1961) 1547.
[11] T. B. PIERCE AND P. F. PECK, *Analyst*, 86 (1961) 580.
[12] B. A. LOVERIDGE AND A. F. OWENS, *At. Energy Res. Estab. (Gr. Brit.), Rept.*, R 3945.
[13] T. B. PIERCE AND P. F. PECK, *Analyst*, 87 (1962) 369.
[14] D. F. PEPPARD, G. W. MASON, J. L. MAIER AND W. J. DRISCOLL, *J. Inorg. Nucl. Chem.*, 4 (1957) 334.
[15] T. B. PIERCE AND P. F. PECK, *Nature*, 195 (1962) 597.
[16] D. F. PEPPARD, J. R. FERRARO AND G. W. MASON, *J. Inorg. Nucl. Chem.*, 7 (1958) 231.
[17] S. W. MAYER AND E. R. TOMPKINS, *J. Am. Chem. Soc.*, 69 (1947) 2866.
[18] M. LEDERER AND S. KERTES, *Anal. Chim. Acta*, 15 (1956) 226.
[19] T. B. PIERCE AND P. F. PECK, *J. Chromatog.*, 6 (1961) 248.
[20] E. CERRAI AND C. TESTA, *J. Chromatog.*, 8 (1962) 232.

THE SEPARATION OF THE RARE EARTHS BY PARTITION CHROMATOGRAPHY WITH REVERSED PHASES

PART II. BEHAVIOUR OF INDIVIDUAL ELEMENTS ON HDEHP–CORVIC COLUMNS

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In Part I of this series[1] it was suggested that a useful separation of the rare earths might be achieved if the properties of certain complexing agents, known to provide large separation factors for adjacent lanthanides in liquid–liquid systems, (*e.g.* di- (2-ethylhexyl) orthophosphoric acid, HDEHP) were exploited by chromatographic elution[1]. A system was therefore developed in which HDEHP was retained on a poly-(vinyl chloride/vinyl acetate) copolymer (Corvic) and the resulting solid used in columns for chromatographic determinations. It was found that this column material could be conveniently prepared with reproducible elution characteristics and that sharp elution peaks could be obtained if columns were run at 60°. A third power dependency of $(1/R_F - 1)$ upon the hydrogen ion concentration of the eluting phase showed that the behaviour of HDEHP retained on Corvic had some similarities with that when the reagent was dissolved in an organic diluent such as toluene. This system is known to give good separation factors for adjacent lanthanides[2], but a wider assessment of the potentialities of HDEHP–Corvic columns for the chromatographic separation of the rare earths would be possible only if the separation factors of all elements of the series, achieved by elution from these columns, were investigated. Consequently, to determine the effect of transferring HDEHP from a liquid to a solid phase, a number of elutions and liquid–liquid extractions of all the elements from lanthanum to lutetium were carried out.

EXPERIMENTAL

The chromatographic apparatus, reagents and techniques were similar to those already described[1], the columns being run at temperatures of 60°. HDEHP–Corvic mixtures as made up, were found to have a capacity of 0.32 mequiv./g by saturation with metal and were used for elutions between 1 and 10 days after preparation.

For solvent extraction 20 % v/v solutions of purified HDEHP in redistilled toluene were used and equilibrations carried out at 25° ± 0.1°. Each of the two phases contacted during the extraction process was pre-equilibrated with the other at this temperature and 10 ml of both solutions were pipetted into a thermostated centrifuge tube. After active lanthanide tracer had been added in not more than 0.01 ml of

solution, the phases were mixed by stirring briskly for 20 min and then separated by centrifugation. To confirm that equilibrium had been attained organic phases containing extracted tracer were re-equilibrated with fresh perchloric acid to enable equilibrium to be reached from conditions of metal deficient as well as metal rich aqueous phase. No attempt was made to maintain the ionic strength of the aqueous phase constant and acid solutions were made up by diluting concentrated acid to the appropriate value.

2 ml of each solution were carefully pipetted off to enable the distribution of radioactivity to be assayed and the acid strength of the aqueous phase obtained by titration against standard alkali. Experimental results were plotted on a graph of log of the distribution ratio (log D) *versus* log of the hydrogen ion concentration of the aqueous phase for liquid–liquid determinations, and a graph of $R_M = \log (1/R_F - 1)$ *versus* log of the hydrogen ion concentration of the eluting phase for elutions. The best straight lines through the points for each individual element were found by the method of least squares, and slopes and intercepts were calculated.

RESULTS

Perchlorate system

Since aqueous phases were made up by dilution of concentrated acid with water, an increase in the hydrogen ion concentration of the aqueous phase was associated with an increase in the concentration of the anion of the acid. In order to reduce the effect of complexes that might be formed between the rare earths and this anion, a series of elutions was carried out using perchloric acid as aqueous phase. As in Part I of this series[1] it was considered that batch extraction methods might be unsuitable for determining distribution ratios when HDEHP–Corvic mixtures were employed as solid phase, since shaking might cause the complexing agent to strip from the polymer. The results obtained for the elution of all the lanthanides from HDEHP–Corvic columns by aqueous perchloric acid are presented on a plot of R_M against the log of the hydrogen ion concentration of the eluting phase in Fig. 1. It can be seen that in

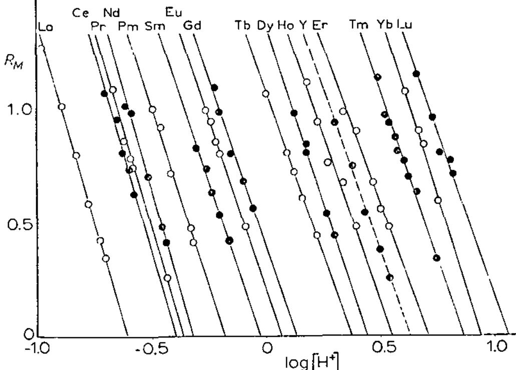

Fig. 1. Elution of the lanthanides from HDEHP–Corvic columns with perchloric acid.

all cases a linear relationship is found, the average slopes of all the lines being -3.13 ± 0.29, which is close to the expected value[2] of -3.0. Experimental results for yttrium are also included. If it is assumed that the elution of the rare earths from

columns of a complexing agent immobilised on a solid support is accounted[1] for by the following equation:

$$R_M = \log A_s/A_m + \log K^{11} + n \log [\mathrm{HL}]_0 - n \log [\mathrm{H}^+] \quad (1)$$

it can be seen that for the elution of two elements from columns of similar characteristics (*i.e.* columns for which A_s, A_m and $[\mathrm{HL}]_0$ are the same) at a given R_M value:

$$\log K_1{}^{11} - \log K_2{}^{11} = n \log [\mathrm{H}^+]_1 - n \log [\mathrm{H}^+]_2$$

subscripts 1 and 2 referring to the values obtained for the two different elements. A plot of 3 log $[\mathrm{H}^+]$ at $R_M = 1.0$ is shown for the rare earths in Fig. 2 from which it can be seen that the value of the intercept increases linearly with the atomic number of the elements eluted, the slope of the line corresponding to an average separation factor for adjacent rare earths of 2.44 ± 1.15.

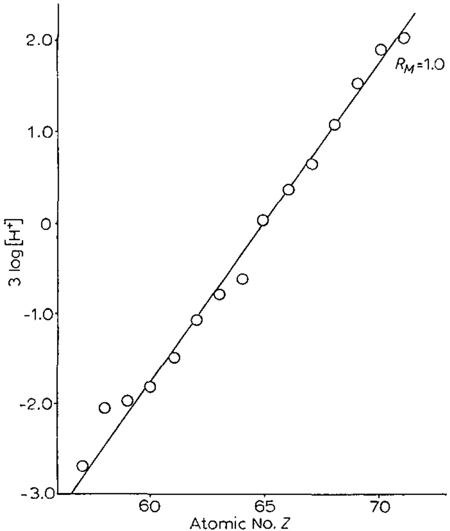

Fig. 2. Plot of 3 log $[\mathrm{H}^+]$ at $R_M = 1.0$ against atomic number for perchlorate elutions.

This compares well with the average value of 2.40 ± 0.87 found for the extraction of rare earths from aqueous perchloric acid, by a solution of HDEHP in toluene[3], but clearly separation factors for adjacent pairs vary considerably throughout the series.

Hydrochloric acid

Hydrochloric acid was used in place of perchloric acid as aqueous phase for a series of elutions, since the chloride systems could prove more acceptable than the perchlorate for a number of practical separations. Results of the hydrochloric acid elutions are given in Fig. 3 from which it can be seen, once more, that consistant results are obtained, the average slope of the best straight lines through the points being -2.73 ± 0.37. Again, however, the aqueous phase was made up from concentrated acid by dilution with water, and therefore the chloride ion concentration increased as the acidity of the aqueous phase was increased.

For comparison the rare earths were extracted from aqueous hydrochloric acid into a solution of HDEHP in toluene at 25°, and the results of these extractions are given in Fig. 4, on a plot of log D, (log of the distribution ratio), against the log of the

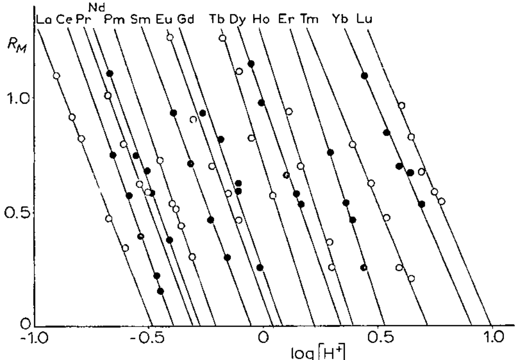

Fig. 3. Elution of the lanthanides from HDEHP–Corvic columns with hydrochloric acid.

hydrogen ion concentration of the aqueous phase, the average slope of the lines being -3.57 ± 0.25.

Since the equation, equivalent to eqn. (1) for liquid–liquid extractions when phases of the same volumes are used is:

$$\log D = \log K_L + n \log [HL]_0 - n \log [H^+]$$

where K_L is a constant and the subscript o refers to species present in the organic phase, a graph of 3 log $[H^+]$ at a given value of log D, against atomic number for the rare earths should give a measure of the separation factors for adjacent elements. This is shown in Fig. 5, together with the value of 3 log $[H^+]$ at which $R_M = 1$ in the

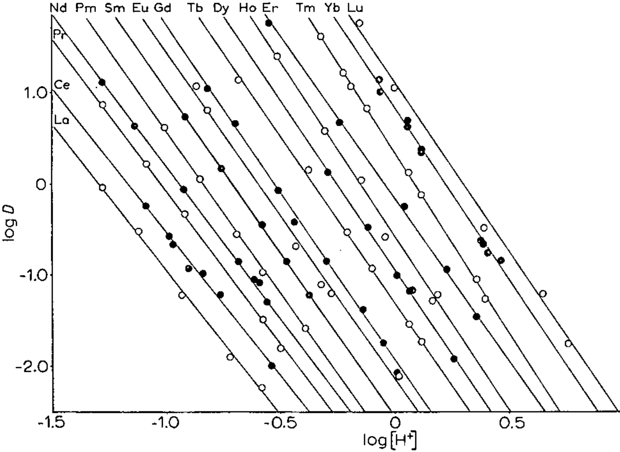

Fig. 4. Distribution of the lanthanides between aqueous hydrochloric acid and a solution of HDEHP in toluene.

chloride system. The average separation factors for the liquid–liquid and liquid–solid systems are 2.24 ± 0.42 and 2.11 ± 0.57 respectively.

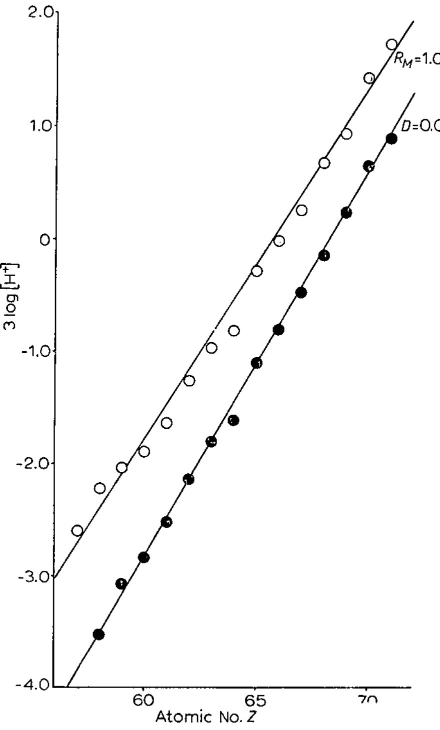

Fig. 5. Plot of 3 log $[H^+]$ at R_M = 1.0 and log D = 0 against atomic number for hydrochloric acid elutions and extractions. ● refers to extractions and ○ to elutions.

DISCUSSION

HDEHP-Corvic mixtures were developed as a column material, suitable for chromatographic operation, since it was hoped that the good separation factors, found for adjacent rare earths in liquid–liquid systems when the alkyl-phosphoric acid was dissolved in an organic diluent such as toluene, would be unaltered when the HDEHP was immobilised on a solid supporting material. For this to occur, the effect of the other constituents of the non-aqueous phase upon HDEHP–rare earth equilibria must be small, or at least produce systematic differences in the K^{11} values when the complexing agent is transferred from a liquid to a solid phase, so that the ratios of the K^{11} values of the elements, (the separation factors) do not vary. The average separation factor for adjacent members of the rare earth series, calculated from perchlorate elutions, is in fact very near to that found for the liquid–liquid perchlorate system (2.44 ± 1.15 compared with 2.40 ± 0.87) thus indicating that transference of the HDEHP from a liquid to a solid phase does not diminish the possibility of obtaining good rare earth separations with the reagent. Rigorous comparison between liquid–liquid and liquid–solid results quoted in this paper and elsewhere[3] for the same aqueous phase is not possible, since, in order to obtain sharp elution peaks, chromato-

graphic elutions were carried out at 60° whereas the more convenient temperature of 25° was used for liquid–liquid extractions and the acid ranges over which both series of experiments were carried out for the same element were different, thus resulting in different acid anion concentration in the two cases. However, if varying the concentration of the perchlorate ion in the aqueous phase has little effect on the distribution of the rare earths between the aqueous and non-aqueous phase, and the effect of temperature on the extraction of the rare earths is either small or similar for all the elements, then the individual separation factors for adjacent pairs of elements should be nearly equal for the liquid–liquid, and for the liquid–solid systems, provided that the alkyl phosphoric acid behaves in the same manner in both cases.

In Fig. 6 the values of 3 log $[H^+]$ at $R_M = 1.0$ for perchlorate elutions are plotted against atomic number for all the elements from lanthanum to lutetium as in Fig. 2, together with the values of 3 log $[H^+]$ at which $\log D = 0$ in the liquid–liquid system,

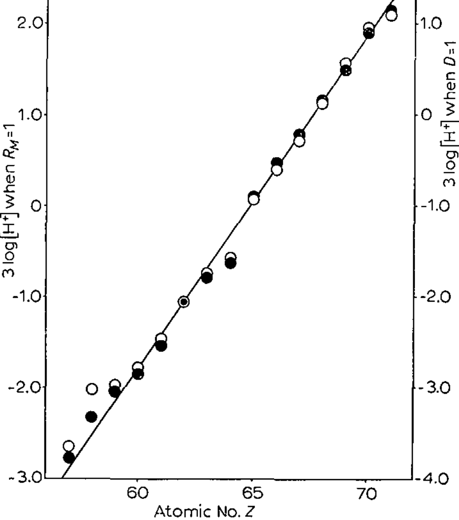

Fig. 6. Comparison of separation factors found from perchlorate extractions and elutions. ● refers to liquid–liquid results and O to liquid–solid results.

with the scale displaced by the average difference between 3 log $[H^+]$ when $R_M = 1.0$ and 3 log $[H^+]$ when $\log D = 0$ for each element. From this it can be seen that the relative positions of the points derived from solvent extraction results, correspond well with those taken from elution measurements, the largest difference occurring in the case of cerium, which shows a smaller separation from praseodymium in the liquid–solid than in the liquid–liquid system. Thus, generally, similar separation factors are obtained in the two systems, which is of interest, since liquid–liquid measurements are derived from equilibrium determinations with the HDEHP dissolved in toluene, whereas for elutions, the aqueous phase flowed past the reagent which was immobilised on a solid.

Further, the results suggest that it should be possible to predict the behaviour of elements on these columns from liquid–liquid results, if the position of the elution maximum for one element is known at a given acidity, together with the liquid extraction data for this and the other elements to be eluted.

The linear relationship between 3 log [H^+] at R_M = 1.0 and atomic number indicates that a mixture of all the elements from lanthanum to lutetium could be separated by elution from HDEHP-Corvic columns, and that elements would be eluted in the order of increasing atomic number, that is to say in the order which is the reverse of that found in conventional cation-exchanger–aqueous complexing agent procedures. Yttrium, if it were to fall on the line shown in Fig. 2, would have to have an atomic number of 67.4 which is in good agreement with the value of 67.5 found for the liquid–liquid system[3].

When hydrochloric acid is used in place of perchloric acid, any chloro-complexes formed in the aqueous phase would be expected to favour a decrease in the distribution of the rare earths into the organic phase, and this effect, if appreciable, would be apparent in determinations, such as those quoted in Figs. 3 and 4, where the chloride concentration of the aqueous phase was changing. Plots of R_M and log D against 3 log [H^+] were found to be linear, within the limits of experimental error, over the acid ranges used for each element, but separation factors were generally smaller when chloride, as opposed to perchlorate was the anion of the acid, the average separation factors being 2.24 $\pm$ 0.42 and 2.11 $\pm$ 0.57 for the liquid–liquid and liquid–solid systems respectively. This compares with the values of 2.40 $\pm$ 0.87 and 2.44 $\pm$ 1.15 quoted above for the equivalent values in the perchlorate system, and the value of 2.5 found for the chloride system at constant chloride concentration[2].

The separation factors for adjacent pairs of rare earths calculated from the elution data presented in Figs. 1 and 3 are given in Table I, together with values found for liquid–liquid systems (Fig. 4 and ref. 3) and for ion-exchange elutions[4].

It can be seen that the most difficult mixtures of elements to separate from one another by elution from HDEHP-Corvic columns will be cerium–praseodymium–neodymium, and europium-gadolinium, and this has already been found to be the case in practice[5]. With the exception of the cerium–praseodymium separation, and perhaps the praseodymium–neodymium separation, separation factors found when

TABLE I

SEPARATION FACTORS FOR THE RARE EARTHS IN SOME HDEHP AND ION-EXCHANGE SYSTEMS

Separation	*HDEHP*				*Dowex 50*		
	Perchlorate system		*Chloride system*		*Glycolate at 87°*	*α-Hydroxy-isobutyrate at 87°*	*Lactate at 20°*
	Elutions	*Extractions*	*Elutions*	*Extractions*			
La–Ce	4.7	3.0	2.4	2.4	2.6	2.2	2.1
Ce–Pr	1.3	2.1	1.5	2.8	1.5	1.6	2.04
Pr–Nd	1.4	1.4	1.4	1.7	1.4	1.57	1.38
Nd–Pm	2.1	2.2	1.9	2.1	1.26	1.61	1.35
Pm–Sm	2.6	3.1	2.3	2.4	1.29	1.82	1.32
Sm–Eu	1.8	1.9	2.0	2.2	1.22	1.6	1.20
Eu–Gd	1.5	1.4	1.4	1.6	1.06	1.4	1.04
Gd–Tb	5.2	5.0	3.3	3.2	1.3	2.2	1.50
Tb–Dy	1.9	2.1	1.9	2.0	1.39	1.88	1.65
Dy–Ho	1.8	1.9	1.9	2.1	1.2	1.63	1.43
Ho–Er	2.7	2.3	2.7	2.1	1.5	1.23	1.60
Er–Tm	3.3	2.5	1.8	2.5	1.4	1.30	1.54
Tm–Yb	2.2	3.1	3.1	2.5	1.5	1.33	1.54
Yb–Lu	1.8	1.9	2.0	1.8	1.32	1.36	1.40
Average	2.4	2.4	2.1	2.2	1.43	1.62	1.51

HDEHP is immobilised on Corvic, are not appreciably worse than the best figure given in Table I for ion-exchange separations, and in most cases are very much better.

Separation factors given in Table I derived for elutions and extractions of pairs of elements by the same acid suggest that the effect that immobilising the complexing agent on Corvic has on the relative positions of the rare earth–HDEHP equilibria is small, and it is therefore likely that similar figures to those quoted above would be obtained in reversed phase systems where the HDEHP was retained on another inert support. Separation factors have been reported for a number of pairs of rare earths after elution by hydrochloric acid from columns of HDEHP retained on cellulose powder[6]. The values obtained, (La–Ce 2.35; Nd–Pm 2.10; Eu–Gd 1.65; Gd–Tb 3.85; Tm–Yb 3.33), cannot be compared exactly with the figures given in Table I because of a difference in operating conditions but are clearly similar to them.

A disadvantage of the reversed phase system described in this paper compared with conventional ion-exchangers for the separation of macro quantities of rare earths is provided by the relatively low capacity of HDEHP–Corvic columns for the extraction of metals. Whilst it has been found that the capacity of the column material used in these determinations (0.32 mequiv./g) could be considerably improved by adding more phosphoric acid to the polymer[1] and it is possible that higher loadings could be achieved with other support materials[7], the acid–base binding capacity of $\sim$ 3 mequiv./g indicates that even under the most favourable conditions the capacity of HDEHP mixtures cannot equal that of suitable ion-exchangers ($\sim$ 5 mequiv./g). Nevertheless, the reliability of the system, the order of elution of the elements, the simplicity of the eluting phase and the generally higher separation factors for adjacent elements has resulted in the reversed phase partition technique providing a more satisfactory means for the routine separation of rare earths, prior to analytical determination, in several cases.

ACKNOWLEDGEMENT

The authors wish to thank Mr. R. HALLETT for providing a computer programme for the method of least squares.

SUMMARY

The elements lanthanium, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, ytterbium and lutetium have been eluted by perchloric and hydrochloric acid of different strengths from columns of di-(2-ethylhexyl) orthophosphoric acid retained on a poly-(vinyl chloride/vinyl acetate) copolymer. From these results separation factors have been calculated and these are compared with values obtained from solvent extraction determinations with the alkyl-phosphoric acid dissolved in toluene.

REFERENCES

1 T. B. PIERCE AND R. S. HOBBS, *J. Chromatog.*, 12 (1963) 74.
2 D. F. PEPPARD, G. W. MASON, J. L. MAIER AND W. J. DRISCOLL, *J. Inorg. Nucl. Chem.*, 4 (1957) 334.
3 T. B. PIERCE AND P. F. PECK, *Analyst*, 88 (1963) 217.
4 B. K. PREOBRAZHENSKII, *Khim. Nauka i Promy.*, 4 (1959) 521.
5 T. B. PIERCE AND P. F. PECK, *Nature*, 195 (1962) 597.
6 E. CERRAI, C. TESTA AND C. TRIULZI, *Energia Nucl.* (*Milan*), 9 (1962) 377.
7 A. G. HAMLIN, B. J. ROBERTS, W. LOUGHLIN AND S. G. WALKER, *Anal. Chem.*, 33 (1961) 1547.

CHROMATOGRAPHY ON PAPER IMPREGNATED WITH ION-EXCHANGE RESINS

PART VIII. SOME TEMPERATURE EFFECTS IN PARTITION AND ION-EXCHANGE PAPER CHROMATOGRAPHY

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INTRODUCTION

Numerous authors have measured and discussed the effect of temperature on R_F values in partition chromatography (for summaries see ref. 1), and it is generally agreed that a constant temperature is essential for reproducible R_F values and that the variation in R_F values due to changes in the temperature are rather small. The effect of temperature on ion exchange equilibria has been studied by KRAUS AND RARIDON[2] over a wide range of temperatures, however no data are available so far for papers loaded with ion-exchange resins. Since in such papers not only the equilibrium constant but also the ratio of solvent to resin may change with temperature, it was decided to investigate the effect of temperature on the R_F values on such ion-exchange papers. Experiments with some partition systems were also carried out in order to permit a comparison of the relative temperature effects.

In addition to the work undertaken on constant temperatures, we also investigated the effect of variations of temperature during development. This was suggested to us by the spectacular success of temperature programmed gas chromatography on the one hand and the statement of RITCHIE[3] that under field conditions without temperature control improved separations were sometimes noted in paper chromatograpy.

TECHNIQUE

The chromatograms were developed in jars 25 cm high and 14 cm diameter, tightly closed with a rubber stopper, which carried a thermometer. Development at 35° and 50° was carried out in a thermostatically controlled oven and a refrigerator was used for development at 4°. The temperature of the laboratory was air-conditioned to 20° (± 1°) for the chromatograms carried out at 20°. The solvents were equilibrated at the temperature of development, *viz.* those for development at 4° were equilibrated in the refrigerator and those for 35° and 50° at the corresponding temperature in the oven.

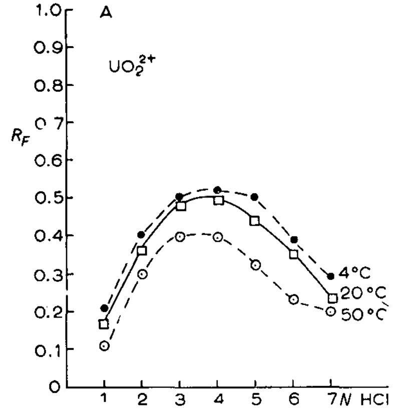
The solvent (aqueous or otherwise) was usually added to the container at the desired temperature and left for several hours until it had acquired the temperature of its surroundings. The paper was then introduced into the container and allowed to

hang from a glass hook in the rubber stopper for at least 30 min and then lowered into the solvent for development. With partition solvents the container also held a 150 ml beaker containing the aqueous phase of the solvent mixture. For experiments with temperature changes during development, the equilibration of the solvent was carried out at a chosen temperature and a different temperature for the development then obtained by placing the colder (or warmer) container into a new ambient temperature. Development was then started after 30 min in the new ambient temperature.

RESULTS

(a) *Ion exchange papers*

Figs. 1 and 2 show the temperature variation of R_F values on the cation resin paper SA-2 (Rohm and Haas) from 4° to 50° plotted against the normality of HCl in Fig. 1, and against the temperature in Fig. 2. Three ions taken at random were studied, namely Cu(II), UO_2^{2+} and Fe(III). It is evident from Fig. 1 that each ion has a different temperature variation, *i.e.* the temperature effect on the ion-exchange equilib-



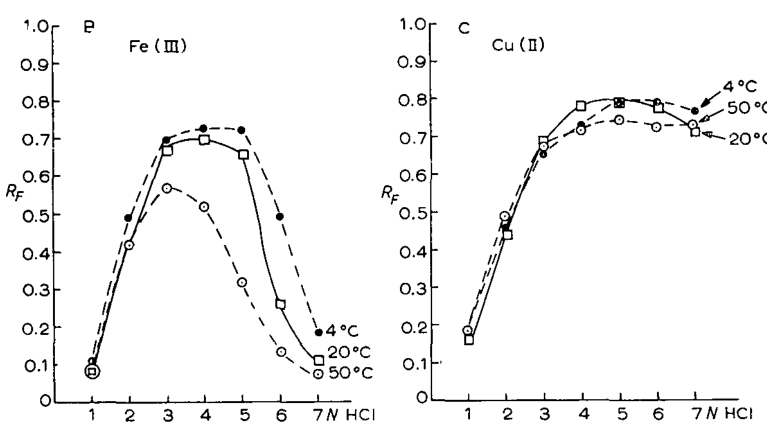


Fig. 1. R_F values of some metal ions on SA-2 cation resin paper plotted against the normality of HCl at 4°, 20° and 50°. (a) Uranyl ion; (b) ferric ion and (c) cupric ion.

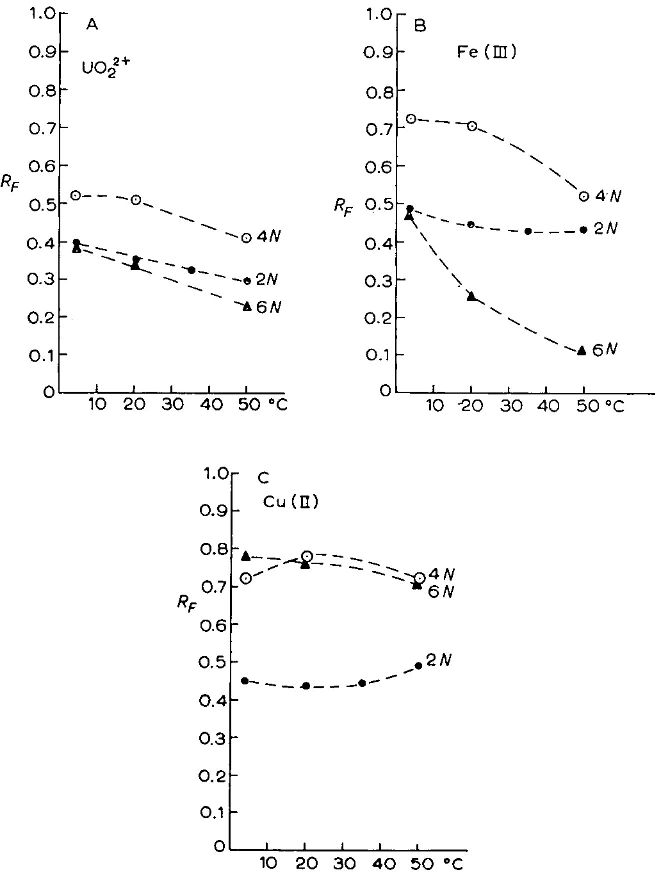


Fig. 2. R_F values of some metal ions on SA-2 cation resin paper developed with HCl. The R_F values are plotted against the temperature for 2*N*, 4*N* and 6*N* HCl. (a) Uranyl ion, (b) ferric ion and (c) cupric ion.

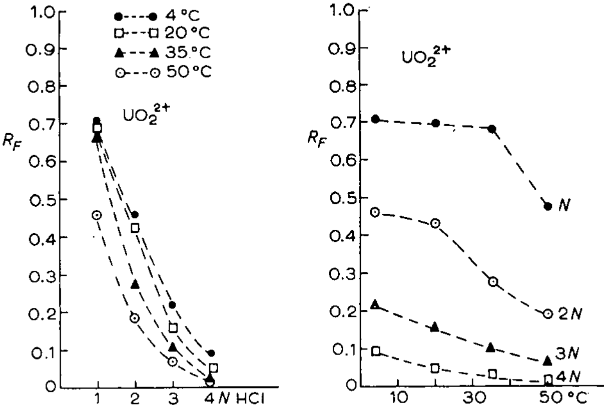


Fig. 3. R_F values of uranyl ion on SB-2 anion resin paper developed with HCl. (a) Plotted against the normality of HCl for 4°, 20°, 35° and 50°; (b) plotted against the temperature for *N*, 2*N*, 3*N* and 4*N* HCl.

rium is probably the preponderant factor. Fig. 2 also shows that except under some conditions, *e.g.* Fe(III) in $5N$ and $6N$ HCl the temperature variation is very small, for example the R_F value of Cu(II) in HCl remains practically constant from 4° to 50°.

Preliminary experiments in which the temperature was varied during development indicated that there is no marked effect due to temperature variation. It seems then that little can be gained by programming, or by raising or lowering of the temperature with these papers.

The behaviours of UO_2^{2+} on the anion resin paper SB-2 (Rohm and Haas) shows a similar trend to that on the cation exchange paper with perhaps a more pronounced temperature effect. It appears that essentially the same results can be expected within the usual range of room temperatures, as is shown in Fig. 3.

(*b*) *Partition chromatography*

Tables I and II show the variation of the R_F values of some inorganic ions in butanol–$3N$ HCl and butanol–$1.5N$ NH_4OH on Whatman No. 1 paper. Similar results with other solvents have been observed previously[1]. We would, however, like to point out that the variations noted are much greater than the variation of the solubility of butanol in water and of water in butanol which varies over this range of 45° by approximately 3 %[4], and hence are probably due to variations of the ratio of the

TABLE I

R_F VALUES OF SOME ANIONS

Solvent: *n*-butanol–$1.5N$ NH_4OH (1:1). Paper: Whatman No. 1. Development: ascending.

Temperature of solvent equilibration	4°	20°	50°	20°	20°
Temperature of development	4°	20°	50°	4°	50° (*3 chromatograms*)
Anions			R_F *values*		
Cl^-	0.08	0.10	0.14	0.07	0.26, 0.15, 0.29
Br^-	0.14	0.16	0.20	0.13	0.37, 0.24, 0.43
I^-	0.22	0.25	0.28	0.21	0.55, 0.38, 0.60
CNS^-	0.37	0.37	0.45	0.32	0.73, 0.53, 0.80

two phases (A_L/A_S) as well as variations in the partition coefficients of the ions. However, all these effects are small. Unexpectedly high variations due to temperature were obtained with partition chromatograms where the solvent was equilibrated at room temperature and the development carried out at 50°. The R_F values obtained can be as much as double those obtained with solvents equilibrated at 50° as shown in Tables I and II. When chromatograms are carried out at a temperature lower than that at which the solvent was equilibrated the effect is minimal.

The general conclusion from these observations is that while the actual temperature for development is not of great importance in paper partition chromatography, temperature variations during development are of consequence.

In ion-exchange paper chromatography neither the actual temperature nor

variations during development will have a great effect within the usual range of room temperatures.

The marked variations obtained when the temperature is changed for development in partition chromatography can be explained by the fact that if the solvent and the aqueous phase are not quite in equilibrium the amount of evaporation from the paper can be considerable, owing to the extremely large surface in relation to its liquid content. The increased movement of ions may be accounted for if we imagine

TABLE II

R_F VALUES OF SOME METAL IONS

Solvent: *n*-butanol–3*N* HCl (1:1). Paper: Whatman No. 1. Development: ascending.

Temperature of solvent equilibration	4°	20°	50°	20°	20°
Temperature of development	4°	20°	50°	4°	50°
Metal			*R_F values*		
Cu(II)	0.16	0.26	0.27	0.17	0.47
Fe(III)	0.18	0.28	0.42	0.19	0.86
Cd(II)	0.66	0.91	0.77	0.66	0.93
Bi(III)	0.50	0.66	0.62	0.50	0.84

that there is continuous evaporation from the liquid front as well as from the surface of the paper, which is then compensated by new solvent moving over the paper. Most publications reporting R_F values at various temperatures show a greater variability of results at room temperature, where variation is greatest, than with temperatures maintained in ovens.

Finally, we should like to raise the question of whether it is in general advisable to carry out paper chromatography at room temperature, which is difficult to maintain at a constant level and where the opening of a door may cause temperature gradients from one side of the development vessel to the other. It may be rather an advantage to work at a given oven temperature *e.g.* 35° where variations can be kept to a minimum.

SUMMARY

The effect of temperature variation in paper partition and ion exchange paper chromatography of some inorganic ions was studied. While the temperature coefficient is rather small, variations of the temperature during development were found to have a considerable effect in partition chromatography but only little effect in ion exchange paper chromatography.

REFERENCES

[1] I. M. Hais and K. Macek, *Handbuch der Papierchromatographie*, VEB Gustav Fischer Verlag, Jena, 1958, p. 142; M. Lederer, *Proc. 2nd Intern. Congr. Surface Activity*, Butterworths, London, p. 506.
[2] K. A. Kraus and R. J. Raridon, *J. Am. Chem. Soc.*, 82 (1960) 3271.
[3] A. S. Ritchie, *J. Chromatog.*, 10 (1963) 281.
[4] A. E. Hill and W. M. Malisoff, *J. Am. Chem. Soc.*, 48 (1962) 925.

Short Communications

Analyse fonctionnelle par chromatographie en couche mince

La chromatographie en couche mince[1] permet la comparaison, par migration différentielle, de quelques microgrammes de produits variés. L'obtention de spots de mêmes R_F sur la même plaque est une condition nécessaire, mais non suffisante, de l'identité de deux produits. Pour augmenter le pouvoir de conviction de telles comparaisons, il est courant de les multiplier sur divers dérivés; par exemple, s'il s'agit d'alcools, on compare également les acétates, etc., soit sur divers adsorbants, soit avec divers systèmes éluants.

Nous désirons attirer l'attention sur un mode opératoire extraordinairement simple qui semble susceptible d'aider considérablement de telles comparaisons, ainsi que de faciliter l'analyse fonctionnelle de micro-quantités d'une substance inconnue. Il s'agit de réaliser les réactions de modification fonctionnelle directement sur les quelques microgrammes de produit servant à la préparation de la chromatoplaque. Deux modes opératoires sont possibles:

Premier mode opératoire

On dépose, au même endroit de la plaque, une micro-goutte du produit à analyser et une ou plusieurs micro-gouttes de réactif spécifique; pour améliorer le mélange des deux, on a intérêt à déposer une micro-goutte du réactif avant et une après le produit à analyser; après quelques minutes de contact, on élue comme d'habitude et on révèle. Par exemple, ayant à identifier dans une essence une fraction paraissant

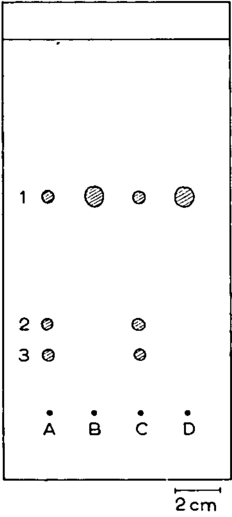


Fig. 1. Époxydation par l'acide *p*-nitroperbenzoïque. A et B: α-terpinéol pur; C et D: le produit à identifier. 1 = α-terpinéol; 2 et 3 = époxydes de l'α-terpinéol.

identique à l'α-terpinéol, nous avons déposé sur une plaque (Fig. 1) de l'α-terpinéol pur (taches A et B) et le produit à identifier (taches C et D); sur les taches A et C, nous avons déposé une solution d'acide *p*-nitroperbenzoïque[2] dans l'éther (solution à 1 %) — après quelques minutes, nous avons procédé à l'élution. Dans ces conditions, l'α-terpinéol donne deux spots nouveaux dûs à des époxydes (d'après leur polarité), bien séparés par la chromatoplaque. On renforce donc considérablement la présomption d'identité du produit, d'abord par la coincidence des R_F des époxydes et d'autre part, par celle de leurs colorations avec divers révélateurs.

Cette première technique avait d'ailleurs déjà été proposée par MILLER ET KIRCHNER[3], mais elle n'a guère été mentionnée depuis. Les désavantages en sont:

(1) l'impossibilité d'utiliser une réaction un peu lente,

(2) l'élution centrifuge du premier produit déposé par la solution du second, ce qui conduit nécessairement à un mélange et à une réaction incomplets.

Deuxième mode opératoire

Ces inconvénients sont évités si l'on réalise la réaction dans le tube capillaire servant à déposer les solutions sur la chromatoplaque.

On remplit, à la longueur désirée, un capillaire de la solution du produit à identifier et un autre de la solution du réactif. Par capillarité, on transfère l'un dans l'autre, puis on mélange par déplacement dans le tube. On peut laisser le mélange en contact aussi longtemps que l'on veut: le solvant, même volatil, s'évapore peu d'un capillaire. Si l'on désire chauffer, on scelle à la microflamme les deux extrémités du capillaire. On peut ensuite au moment choisi utiliser directement le contenu du capillaire pour le porter sur la chromatoplaque. La comparaison avec un témoin traité dans les mêmes conditions est d'autant plus probante que la réaction utilisée est *moins* univoque: plus nombreux sont les produits formés simultanément, et séparés sur la chromatoplaque, plus est convaincante l'identification ou la différenciation.

Dans le cas d'un produit inconnu, on peut aussi effectuer rapidement sur des quantités minimes des essais de réactions caractéristiques d'un groupe fonctionnel; on vérifie la présence du groupe fonctionnel suspecté par la variation de la polarité et l'apparition de taches nouvelles sur la plaque.

D'innombrables réactions sont utilisables dans ces modes opératoires, parfois plusieurs à la suite dans le même tube. La seule limitation apparente est la nécessité d'utiliser un réactif en solution et ne donnant pas de produit de décomposition interférant avec la chromatographie. Mentionnons les principales applications possibles, dont nous avons utilisé un certain nombre: oxydations par l'acide chromique, l'acide *p*-nitroperbenzoïque, l'hypobromite de sodium, les tétroxydes d'osmium ou de ruthénium; réductions par les hydrures mixtes, le chlorure chromeux; saponification par la potasse; acétylation par l'anhydride acétique et la pyridine; méthylation par le diazométhane; déshydratation par l'acide sulfurique concentré ou l'oxychlorure de phosphore avec la pyridine; désamination par l'acide nitreux; équilibrations par un acide fort; synthèses diéniques, etc. Dans tous les cas, il est inutile de procéder à l'isolement des produits de réaction. Le mélange réactionnel est directement appliqué sur la plaque.

Avant de conclure, il nous paraît indispensable de tenir compte du postulat de MARTIN[4] qui semble en contradiction avec l'analyse fonctionnelle sur chromatoplaque. En effet, selon ce postulat, le R_M d'une substance $[R_M = \log (1/R_F - 1)]$

est égal à la somme des R_M de ses groupements fonctionnels plus une constante pour le système solvant; ce qui revient à dire que deux substances ayant les mêmes R_F forment des dérivés ayant également les mêmes R_F et que la formation de dérivés n'ajoute rien à la caractérisation.

Il n'est pas dans notre intention de discuter le postulat de MARTIN, maintes fois vérifié en chromatographies sur papier et en couche mince[4]. Nous voudrions montrer simplement qu'en pratique, ce postulat ne diminue nullement l'intérêt de la méthode que nous préconisons: dans l'analyse fonctionnelle sur chromatoplaque, deux substances à mêmes R_F se différencieront au moins par leurs différences de réactivité vis-à-vis des réactifs utilisés ou par les colorations de leurs dérivés, traités par divers révélateurs. Par exemple, le géraniol (2 doubles liaisons) et le citronnellol (1 double liaison), à R_F très voisins, se sépareront facilement par époxydation, le premier formant un dioxyde, le deuxième un monoxyde à R_F nettement différent; le géraniol et l'α-terpinéol, difficiles à distinguer par leurs R_F, se différencient nettement par l'acétylation pyridinée qui laisse intact l'α-terpinéol, ou la déshydratation par le mélange $POCl_3$–pyridine qui ne touche pas le géraniol.

En conclusion, l'analyse fonctionnelle par chromatographie en couche mince nous semble une méthode de caractérisation sûre qui mérite l'emploi le plus courant tant en recherche que dans les laboratoires d'enseignement, surtout jointe à l'utilisation des plaques sélectives[5].

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1 J. G. KIRCHNER, J. M. MILLER ET G. J. KELLER, *Anal. Chem.*, 23 (1951) 420.
2 M. VILKAS, *Bull. Soc. Chim. France*, (1959) 1401.
3 J. M. MILLER ET J. G. KIRCHNER, *Anal. Chem.*, 25 (1953) 1107.
4 J. GREEN ET S. MARCINKIEWICZ, *J. Chromatog.*, 10 (1963) 35.
5 C. B. BARRETT, M. S. DALLAS ET F. B. PADLEY, *Chem. Ind. (London)*, (1962) 1050.

Reçu le 19 avril 1963

J. Chromatog., 12 (1963) 94–96

Application du détecteur à capture électronique en chimie des radiations

La distribution finale des produits radiolytiques stables résultant de l'irradiation de composés par des rayonnements ionisants est déterminée en partie par des réactions radicalaires. Une technique courante en chimie des radiations consiste à intercepter les radicaux libres au fur et à mesure de leur formation et à observer les variations des rendements en produits finaux en l'absence de réactions radicalaires. A cet effet on ajoute au système irradié un composé ayant une affinité élevée pour les radicaux et susceptible d'entrer en compétition avec les réactions de recombinaison, de disproportionnement et d'arrachement d'hydrogène qui sont les manifestations les plus courantes de la réactivité des radicaux libres.

L'iode est fréquemment employé comme intercepteur de radicaux. La réaction d'interception:

$$R^{\bullet} + I_2 \longrightarrow RI + I^{\bullet}$$

a une énergie d'activation inférieure à 1 kcal/mole et conduit à la formation d'un iodure organique stable. La mesure de la consommation d'iode au cours de l'irradiation donne une indication sur le rendement total en radicaux libres, mais la détermination qualitative et quantitative de la distribution des iodures, reflet du "spectre radicalaire" de la radiolyse, est d'un enseignement beaucoup plus riche.

Le problème analytique a été discuté par DAUPHIN[1]. La séparation d'un mélange d'iodures organiques peut être réalisée aisément par chromatographie en phase gazeuse, mais les concentrations très faibles des iodures résultant de l'interception de radicaux sont généralement inférieures aux limites de sensibilité des cellules à conductivité thermique. C'est pourquoi les spectres radicalaires des hydrocarbures irradiés ont été établis jusqu'à présent à l'aide d'iode radioactif. L'iode est marqué par ^{131}I et les rendements des iodures organiques sont déduits de la mesure de la radioactivité des fractions séparées par radiochromatographie en phase gazeuse[2] ou par distillation fractionnée[1]. Cette dernière méthode nécessite après irradiation l'addition d'entraîneurs c'est-à-dire de quantités macroscopiques des divers iodures susceptibles d'être présents dans le mélange.

L'emploi d'iode radioactif n'est pas sans inconvénients. ^{131}I est livré sous forme d'une solution d'iodure de sodium dont on doit libérer I_2. Les manipulations qui mettent en jeu des quantités parfois notables de ^{131}I peuvent présenter un certain danger. Aux faibles doses d'irradiation, l'effet du rayonnement de ^{131}I sur le système étudié peut devenir relativement important (surtout par l'absorption du rayonnement β) et s'ajouter à celui de l'irradiation externe, ce qui entraîne une incertitude dans l'évaluation de la dose réellement reçue ainsi que dans l'interprétation des résultats.

Nous avons contourné ces difficultés et évité l'emploi d'iode radioactif en associant à une colonne chromatographique un détecteur à capture électronique qui semble être l'instrument idéal pour l'analyse d'un mélange d'iodures organiques présents en très faibles concentrations dans un hydrocarbure. Ce détecteur dont le fonctionnement a été étudié par LOVELOCK[3-5] et LANDOWNE[6], est constitué par une chambre d'ionisation contenant une source radioactive (250 mC de tritium adsorbé sur un support métallique) reliée à la cathode, le tube d'arrivée du gaz vecteur servant d'anode. Une tension continue de 90 V est appliquée entre les électrodes. Le courant d'ionisation dû au gaz vecteur seul (azote très pur) est de $3 \cdot 10^{-9}$A. L'introduction dans la chambre d'une substance possédant un atome ou un groupement fonctionnel à affinité électronique élevée (atome ou groupement dits "électrophores") entraîne une diminution du courant d'ionisation, proportionnelle au nombre d'électrons captés.

Le détecteur à capture électronique est particulièrement sensible aux iodures organiques, l'affinité électronique de l'iode (3.063 eV) étant nettement supérieure à l'énergie de la liaison C–I (2.48 eV). La limite de concentration décelable est $10^{-12}M$. A titre d'exemple, pour l'iodure d'éthyle la quantité minimale détectable est de $0.16 \cdot 10^{-9}$ g (correspondant à une sensibilité du détecteur $6.6 \cdot 10^{-14}$ mole/sec) et la limite supérieure du domaine de réponse linéaire $14 \cdot 10^{-9}$ g. La réponse du détecteur

aux hydrocarbures qui constituent la majeure partie des mélanges analysés est faible ou nulle, ce qui permet la discrimination de petites quantités d'iodures dont le volume de rétention serait proche de celui du solvant. Enfin, la simplicité de la mise en oeuvre est un attrait supplémentaire du détecteur à capture électronique, dont les conditions opératoires optimales n'ont peut-être pas encore été atteintes.

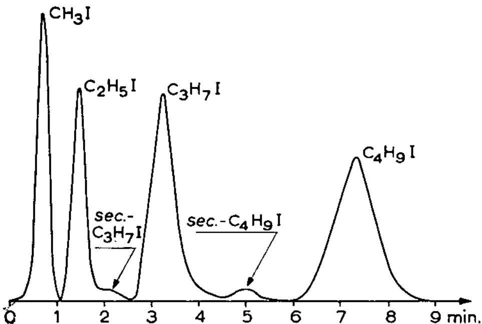

Fig. 1. Analyse chromatographique d'un mélange heptane–iode après irradiation.

Le chromatogramme représenté sur la Fig. 1 est le résultat partiel de l'analyse d'un échantillon de 5 μl d'heptane irradié en présence d'iode (concentration initiale $10^{-3} M$) par les rayons γ de ^{60}Co (dose $4.5 \cdot 10^{19}$ eV/cm^3). Les conditions de la chromatographie ont été les suivantes: colonne de 1 m, phase stationnaire tricrésylphosphate à 15 % sur célite, débit du gaz vecteur 40 cm^3/min, température 60°.

La distribution des iodures a ainsi été déterminée pour plusieurs hydrocarbures et les résultats concordent généralement avec ceux obtenus à l'aide d'iode radioactif.

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[1] J. DAUPHIN, *J. Chim. Phys.*, 6 (1962) 1196.
[2] J. P. ADLOFF, *J. Chromatog.*, 6 (1961) 373.
[3] J. E. LOVELOCK ET S. R. LIPSKY, *J. Am. Chem. Soc.*, 82 (1960) 431.
[4] J. E. LOVELOCK, *Anal. Chem.*, 33 (1961) 162.
[5] J. E. LOVELOCK, *Anal. Chem.*, 35 (1963) 474.
[6] R. A. LANDOWNE ET S. R. LIPSKY, *Anal. Chem.*, 34 (1962) 726.

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Notes

The design of an automatic absolute flow meter for gas chromatography

The accurate measurement of gas flow and gas flow rates may be regarded as one of the outstanding technical problems in gas chromatography. Absolute values are needed when retention data are to be converted into partition coefficients or other thermodynamic data, and the accurate reproduction of flow rates is a vital requirement for the identification of components by means of retention indices. In large-scale chromatography, the programming of the trapping cycle on the basis of gas flow, rather than time, appears attractive.

This paper concerns the design of a flow meter which is essentially an automatic gas burette*.

The gas pressures at the inlet and outlet of the burette are maintained equal by a feedback loop consisting of a differential manometer and a servo amplifier and motor (Fig. 1). When the outlet of the burette is open to the atmosphere, the complete system acts as a metering pump, pumping gas into the atmosphere without changing its pressure. Two outputs are available:

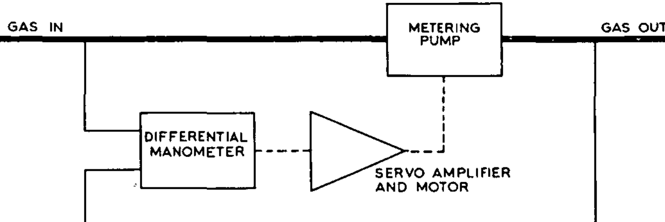

Fig. 1. Block diagram of flow meter.

(1) The number of revolutions of the motor shaft is proportional to the volume pumped through; this will be called the integral output.

(2) The rotational speed of the motor shaft is proportional to the flow rate of the gas; this will be called the differential output.

Description of the instrument

A practical design of the instrument is shown schematically in Fig. 2. The burette consists of a reciprocating piston moving in a cylinder with a net gas volume of, *e.g.*, 50 ml. Movement of the piston is effected by rotation of the drive shaft, which is threaded so that one stroke of the piston corresponds to 100 revolutions of the drive shaft. The revolutions are counted by a counter actuated by a switch and cam on the drive shaft, and the decade transfer on the counter is used to reverse the operation

* Construction is under progress of a prototype instrument, with which the author hopes to be able to obtain actual performance data.

of the burette. The gas connections are changed by means of the two 3-way valves shown, and the drive direction of the piston is changed by means of the bevel gears and the magnetic clutches. Thus the motor always runs in the same direction, and its speed may be used as a differential output.

The manometer is a differential capacitor with a movable centre plate. Similar manometers have been described in the literature[1-3]; these were powered by high-frequency energy, and the movable plate was maintained in the centre position by application of a d.c. potential. The applied d.c. potential is then a measure of the pressure difference across the membrane. The associated electronic equipment can be

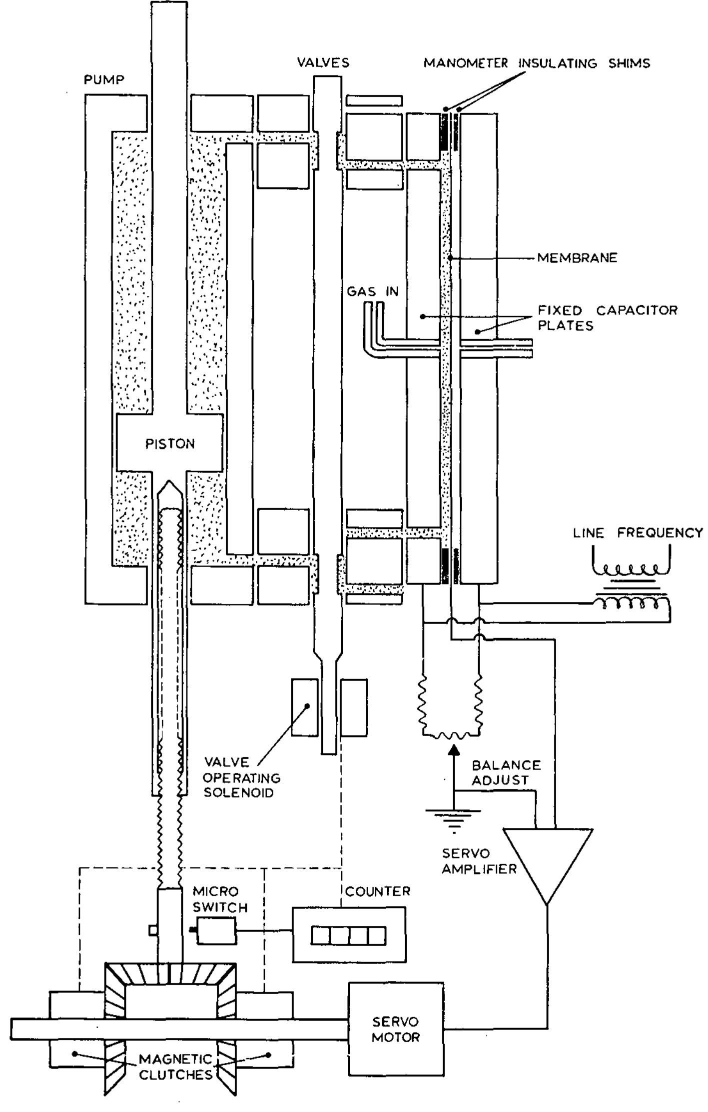

Fig. 2. Schematic representation and lay-out of flow meter.

stable and reliable, and such manometers have a high sensitivity. A different circuit, however, is indicated in Fig. 2. An attractive feature of this circuit is that the signal at the transducer has a frequency, amplitude and phase angle which can be accepted as input by the power amplifier. Proper shielding is necessary, because the source impedance of the transducer is of the order of 20 MΩ, and a simple preamplifier is needed when a servo system with low input impedance (*e.g.* the Honeywell-Brown continuous balance system) is used.

Drift and random errors

When care is taken that the servo system operates in the proportional region, *i.e.* that the amplifier is not driven to saturation, the main causes of error are temperature fluctuations of the burette and frictional changes in the drive system.

The Honeywell-Brown continuous balance system is driven to saturation with an a.c. input of about 500 μV on the grid of the first tube. Calculations have been given for the actual capacitance changes for a properly flexed membrane[1]. For the present purpose this is not necessary; we may obtain an estimate of the output signal by assuming that 10 % of the surface has the maximum displacement calculated by means of the equations given by ROARK[4], and that the remaining surface stays at the central position. With this approximation we may calculate that a pressure difference of 1 mm H_2O across a stainless steel membrane of 100 μ thickness and 40 mm diameter produces an output voltage of 50 mV, when the fixed plates are mounted with 75 μ spacing and driven at 100 V. Thus a pressure change of 10 μ H_2O will produce saturation of the amplifier. The change in volume of the manometer corresponding to this pressure amounts to *ca.* 1 μl; this is the maximum random error in the integral output caused by frictional changes in the drive system.

Temperature fluctuations will produce random errors with a magnitude $V\Delta T/T$, where T is the absolute temperature, and V the gas volume contained in the burette. At room temperature this amounts to 0.16 ml/°C, when the burette volume is 50 ml. This value is reduced to about 0.1 ml/°C at 250°.

Drift in the integral output can be caused by leakage of gas past the piston or through end seals and valves. For the equipment under consideration, greaseless bearings of metal on metal can be made with clearances of 10 μ or less. If the piston has a length of 1 cm and a circumference of 10 cm, the leak rate will be 10^{-4} ml H_2/h at the maximum pressure difference that may exist in practice. Smaller values are obtained for the end seals and the valves, so that drift caused by leakage should be entirely negligible. A change in the temperature would of course result in a change in the integral output. Changes of the outlet pressure do not affect the volume indication of the instrument, but the weight of the gas delivered per stroke will depend on the pressure.

The switching at the end of a stroke may produce transients in the integral output, but the system will "catch up" with the gas flow in a very short time, after which the output will again represent the true integral.

Noise in the differential output can be derived from the noise in the integral output; it depends, of course, on the frequency of the noise sources and the pass band of the feedback system.

Systematic errors can arise from nonlinearity of the thread on the drive shaft, and deviations from the nominal volume of the burette will give rise to a systematic

absolute error. Both errors depend entirely on mechanical factors; they can be held to very low values, and corrections may be made by means of calibration.

Use of the flow meter as a detector

It is interesting to consider the potential usefulness of the flow meter as a gas chromatographic detector. When the system is connected to a gas chromatograph, the integral output curve may have the shape shown in Fig. 3. When a sample is injected, there will be a peak, as the sample vaporizes and then partly dissolves in the stationary phase. This peak may be partly or completely damped out by the filtering action of the column. Under conditions of linear chromatography the volume

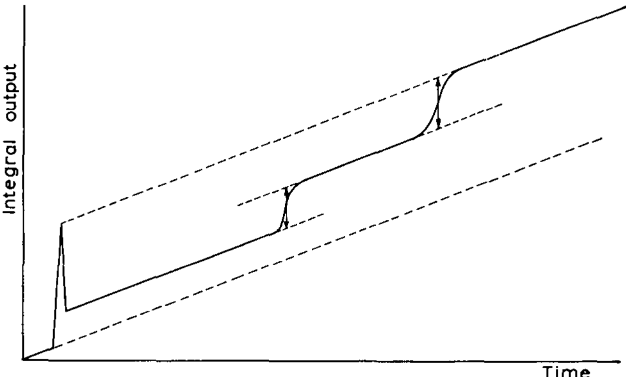

Fig. 3. Theoretical response of flow meter detector for a 1:1 mixture of 2 components having capacity ratios of 2 and 4.

of the sample plug in the gas phase then remains constant as the sample moves through the column. At the column exit, however, the dissolved fraction of the sample re-enters the gas phase, causing a step in the curve with a height proportional to the amount of sample multiplied by $k'/(1 + k')$, where k' is the capacity ratio.

The entire sample should remain in the gas phase while it passes through the flow meter; this condition is met when the instrument is heated to the temperature of the column, and when no adsorption or absorption takes place in the system. As was stated earlier, it is technically feasible to construct the essential parts out of metal, without need for lubrication. The proper choice of alloys will depend on the type of sample to be handled.

The precision and sensitivity of the instrument should be adequate for detection of samples emerging from conventional packed columns. The flow of gas through the column may vary, however, because the presence of the sample affects the viscosity of the gas. In addition, there may be random fluctuations in the flow of carrier gas. Both effects will cause noise in the detector output, which may limit the sensitivity attainable in practice. Where this limit will lie depends to a large extent on the amplitude and frequency of the noise, neither of which can be easily estimated from theoretical considerations.

By means of the arrangement shown in Fig. 4, an output may be obtained which directly represents the sample content of the gas entering the system. Two flow meters are connected in series, with a trap mounted between the two. The first flow meter is

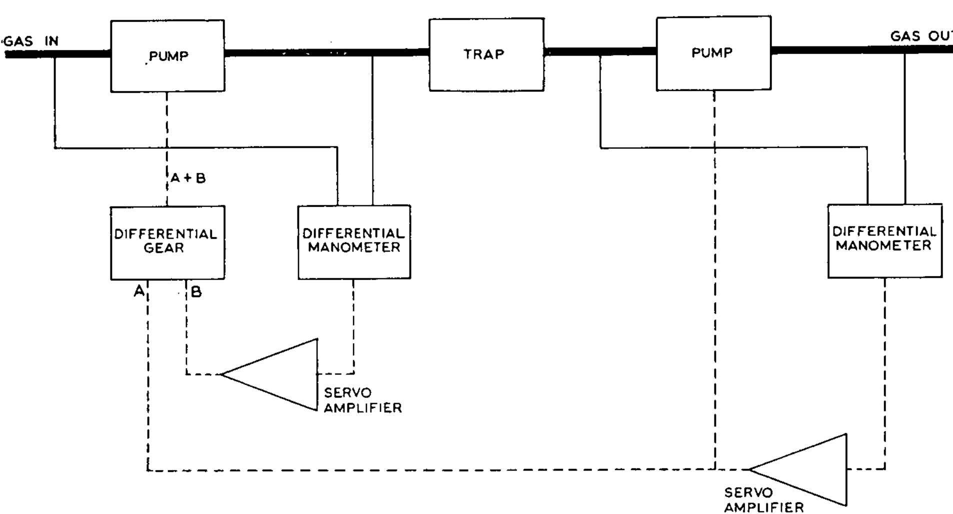

Fig. 4. Schematic diagram of a detector with an integral output directly proportional to the number of moles of sample contained in the incoming gas. Integral outputs: A = pure carrier gas; B = total sample, A + B = total gas flow.

driven via a differential gear, one input of which is driven with a speed corresponding to the flow of the pure carrier gas. When the incoming gas contains a sample component, the first flow meter will lag behind by an amount corresponding to the volume of the sample vapour.

This lag is sensed by the associated manometer, which will cause a correction signal to be given to the flow meter via the second input of the differential gear. Three integral outputs are now available: the flow of pure carrier gas is represented by the signal at A, the sample content at B, and the total gas flow at A + B. It should be noted that this arrangement represents an in-line detector with truly integral characteristics. In addition, the response should be a linear function of the number of moles of sample, when ideal mixing in the gas phase is assumed.

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[1] J. C. LILLY, V. LEGALLAIS AND R. CHERRY, *J. Appl. Phys.*, 18 (1947) 613.
[2] J. J. OPSTELTEN AND N. WARMOLTZ, *Appl. Sci. Res., Sect. B*, 4 (1955) 329.
[3] J. O. COPE, *Rev. Sci. Instr.*, 33 (1962) 980.
[4] R. J. ROARK, *Formulas for Stress and Strain*, 3rd Ed., McGraw-Hill Book Company, New York, 1954, Chap. 10.

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Temperature as a factor in determining order of elution in gas-liquid chromatography of some C_5–C_8 hydrocarbons

The usual practice in gas–liquid chromatography is to work at as low a column temperature as possible when seeking to increase the separation of close-boiling, chemically similar compounds. This is because, in many cases, the relative retention between two solutes increases with decreasing temperature. This view of the situation, however, is a simplified one. PURNELL[1] showed some years ago that, depending on the relative values of vapor pressure and the ratio of the molar heat of solution at infinite dilution to the molar latent heat of vaporization, increasing the column temperature may lead to either an increase or a decrease in resolution for a given pair of solutes. Thus, there may be both an upper and a lower column temperature at which the separation, in terms of the relative retentions, will be the same with a reversal in the order of elution.

Work in this laboratory, connected with the detailed analysis of hydrocarbon fractions isolated from a Fischer-Tropsch product[2], revealed the existence of a complex relationship between the column temperature and the order of elution for some of these hydrocarbons. Fig. 1 is a plot of the logarithm of the retention for eight hydrocarbons, relative to hexane, over the temperature range 40° to 80°. Pure samples of the individual hydrocarbons admixed with hexane were chromatographed

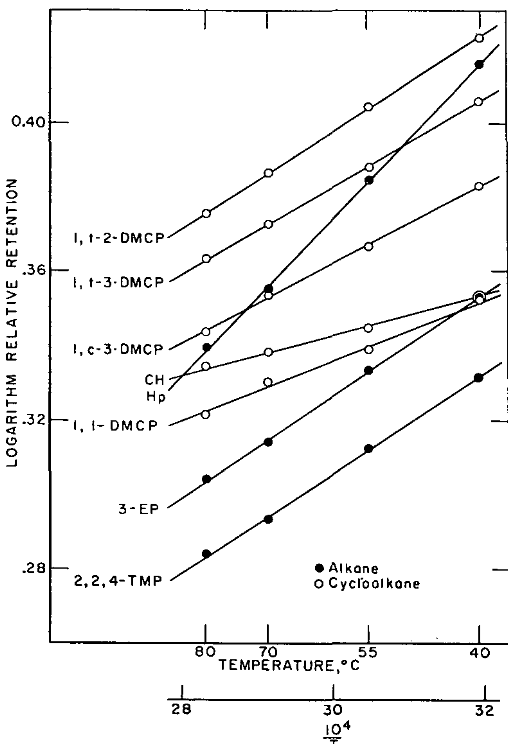

Fig. 1. Logarithm of retention, relative to hexane, as a function of temperature.

on a 26.5 ft. (4.6 mm I.D.) coiled column packed with 79 g of 12% (w/w) tricresyl phosphate on 34–45 mesh Chromosorb P. All the relative retentions are averages of duplicate measurements. Peak retention times were measured with a stopwatch starting from the emergence of the air peak. The helium flowrate was set at 72 c.c./min as measured by a soap-film flowmeter at room temperature.

The tricresyl phosphate used to prepare the column packing when analyzed by mass spectrometry using low-energy ionizing electrons[3] showed the following approximate distribution of molecular weights: 354, trace; 368 (tritolyl phosphate), 39%; 382, 43%; 396, 16%; and 410 (trixylyl phosphate), 2%. Apparently this tricresyl phosphate was prepared from a cresol sample that contained appreciable xylenol. Other tricresyl phosphate samples, including one specifically labelled "for gas chromatography", when analyzed in this fashion showed similar molecular-weight distributions.

A marked change in the order of elution for the hydrocarbons occurs as the column temperature changes. At 40°, the order of elution is 2,2,4-trimethylpentane (2,2,4-TMP), 1,1-dimethylcyclopentane (1,1-DMCP), 3-ethylpentane (3-EP) and cyclohexane (CH), 1-*cis*-3-dimethylcyclopentane (1-*c*-3-DMCP), 1-*trans*-3-dimethylcyclopentane (1-*t*-3-DMCP), heptane (Hp), and 1-*trans*-2-dimethylcyclopentane (1-*t*-2-DMCP). At 80°, the order of elution is 2,2,4-TMP, 3-EP, 1,1-DMCP, CH, Hp, 1-*c*-3-DMCP, 1-*t*-3-DMCP, and 1-*t*-2-DMCP.

For the pair methylcyclohexane–1-*cis*-2-dimethylcyclopentane the relative retention increases from 1.03_4 at 40°, where they are not separable, to 1.05_3 at 80°, where they could be separated sufficiently for analysis. Again, on the same column, the relative retention for the pair cyclopentane–hexane changes from 0.95_4 at 40° to 1.03_3 at 80°.

It is not generally recognized that isolated results showing changes in order of elution with changing column temperature have been reported in several papers. EVERED AND POLLARD[4] showed that 1-nitrobutane, when chromatographed on a squalane-coated packing, elutes before *n*-butyl nitrate at 80°, but that at 139° the order of elution is reversed. SIMMONS *et al.*[5], showed a reversal in order of elution on a squalane-coated capillary between 46° and 106° for the pair 2,2-dimethyl-3-ethylpentane–2,6-dimethylheptane. They also obtained a slightly better separation between 2,5- and 3,5-dimethylheptane at the higher temperature. ROBERTS[6] showed that caryophyllene and farnesene exhibit a reversal in order of elution between 100° and 200° on an Apiezon L packing. BARON AND MAUME[7] reported that several terpene alcohols undergo reversal in order of elution between 130° and 150° when chromatographed on a Reoplex 400 packing. SCOTT[8], in a detailed study of dinonyl phthalate-coated capillary columns, demonstrated that the pair methylcyclohexane–heptane is more easily resolved at 60° than at lower temperatures, partly because the retention ratio increases in going from 0° to 70°. DESTY AND GOLDUP[9] reported better resolution of the octanes on a squalane-coated capillary at 50° than at lower column temperatures.

Thus, various solutes, on a variety of columns, exhibit complicated elution behavior when the column temperature is changed. This may be, therefore, a phenomenon of general interest. As more chromatographic systems are studied in detail, it is to be expected that further examples of this type of behavior will be found. It may be helpful in anticipating other examples to point out that in many cases, both

in this paper (Hp and the DMCP's in Fig. 1, cyclopentane–hexane, and methylcyclohexane–1-*cis*-2-DMCP) and in the literature[6–8], this phenomenon occurred with pairs of compounds consisting of either acyclic and cyclic structures or different ring structures.

Consequently, for solutes that elute close together, column temperature may often be a factor in determining order of elution. The order of elution established experimentally at one temperature may not hold true at a different column temperature, particularly for multicomponent mixtures. In addition, in programmed-temperature gas chromatography, the following situations may arise. If the column temperature is programmed during the run, a separation that would have been achieved at the lower temperature may be partly or wholly canceled out by operating at a higher temperature where a reversal in the order of elution occurs. For compounds eluting close together, the order of elution may depend on the choice of initial and final column temperatures or on the rate of heating of the temperature program.

The authors wish to thank A. G. SHARKEY, JR., for the mass-spectrometric analyses. Reference to specific brands is made to identify materials used and does not imply endorsement by the Bureau of Mines.

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GUS PANTAGES

1 J. H. PURNELL, in D. H. DESTY (Editor), *Vapour Phase Chromatography*, Butterworths, London, 1957, p. 52.
2 B. D. BLAUSTEIN, I. WENDER AND R. B. ANDERSON, *Nature*, 189 (1961) 224.
3 J. H. BEYNON, *Mass Spectrometry and its Applications to Organic Chemistry*, Elsevier, Amsterdam, 1960, p. 428.
4 S. EVERED AND F. H. POLLARD, *J. Chromatog.*, 4 (1960) 451.
5 M. C. SIMMONS, D. B. RICHARDSON AND I. DVORETZKY, in R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, Washington, D. C., 1960, p. 211.
6 J. B. ROBERTS, *Nature*, 193 (1962) 1071.
7 C. BARON AND B. MAUME, *Bull. Soc. Chim. France*, (1962) 1113.
8 R. P. W. SCOTT, *J. Inst. Petrol.*, 47 (1961) 284.
9 D. H. DESTY AND A. GOLDUP, in R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, Washington, D. C., 1960, p. 162.

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The influence of esterifying and acetylating groups on the retention times of amino acid derivatives in gas chromatography

A number of publications have appeared recently reporting studies on the analysis of amino acid mixtures by gas phase chromatographic separation of their more volatile derivatives. In particular the esters[1,2], N-trifluoroacetylated esters[3–8], and N-acetylated esters[9,10] have attracted considerable attention. Each of these reports have in

the main been confined to only one or two derivatives. The present communication reports data showing the relative influence of the various introduced groupings on the gas chromatographic behaviour of a number of the more suitable amino acid derivatives as an aid in the design of systems for the analysis of the amino acids.

The data presented here have been confined to two representative amino acids, *viz.* leucine, and phenylalanine. (The relative retention times for the methyl esters of leucine, phenylalanine and arginine are 0.07, 1.0 and 2.0 respectively. The relative retention times for the N-trifluoroacetyl methyl esters of leucine, phenylalanine, arginine and tryptophan are 0.1, 1.0, 9.0 and 27 respectively.) The amino acids were converted to their ester hydrochlorides by treatment with anhydrous HCl gas in the appropriate alcohol, and then recrystallized from this alcohol. The ester hydrochlorides were in turn converted either into the N-trifluoroacetyl derivative by treatment with trifluoroacetic anhydride[11] or into the acetyl derivative by treatment with acetic anhydride[10].

TABLE I

RETENTION TIMES OF AMINO ACID ESTERS

6 ft. 3/16 in. I.D. glass column packed with 2% neopentyl glycol succinate on Fluoropak 80. Sample size 0.2 μmole.

Ester	*Leucine 135° (min)*	*Phenylalanine 190° (min)*
Methyl	2.2	4.0
Ethyl	2.7	4.6
Propyl	3.8	5.8
Butyl	6.4	8.0
Amyl	10.0	11.1

Two types of column packing were employed, 2% neopentyl glycol succinate on Fluoropak 80 (The Fluorocarbon Co.) which was found most suitable for the amino acid esters and 2% neopentyl glycol succinate on Chromosorb W (acid washed, 80–100 mesh) which was used for the acetylated esters. Nitrogen was the carrier gas flowing at 60 c.c./min and analysis of the effluent gas was by means of a hydrogen flame detector. The injection port and detector were at the same temperature as the column.

TABLE II

RETENTION TIMES OF N-ACETYLATED ESTERS OF LEUCINE

2 ft. 3/16 in. I.D. stainless steel column packed with 2% neopentyl glycol succinate on Chromosorb W (acid washed, 80–100 mesh). Sample size 0.2 μmole.

Ester	*N-Trifluoroacetylated leucine*		*N-Acetylated leucine*	
	142° (min)	*181° (min)*	*142° (min)*	*181° (min)*
Methyl	1.9	0.5	11.0	1.6
Ethyl	2.1			
Propyl	3.0			
Butyl	4.4	1.0	25.7	3.1
Amyl	6.5			

The influence of ester group on retention times. The retention times obtained for the various esters of leucine and phenylalanine in the form of their free bases[12] are shown in Table I, while Table II shows the corresponding values for the N-acetylated and N-trifluoroacetylated derivatives of leucine esters.

The influence of N-acetylation on retention times. Table III gives the retention times obtained on the same column for the methyl esters and N-acetylated methyl esters of leucine and phenylalanine.

TABLE III

RETENTION TIMES OF THE METHYL ESTERS OF LEUCINE AND PHENYLALANINE AND THEIR N-ACETYLATED DERIVATIVES

6 ft. 3/16 in. I.D. glass column packed with 2% neopentyl glycol succinate on Fluoropak 80. Sample size 0.2 μmole.

	Leucine methyl ester 150° (min)	*Phenylalanine methyl ester 180° (min)*
Free base	1.4	4.0
N-Trifluoroacetylated	4.4	7.2
N-Acetylated	20.5	29.0

All other factors being equal, it is advantageous in gas phase analysis to synthesize derivatives of amino acids with low retention times. The results of our comparative studies of the amino acids have led us to favor the methoxyl radical for esterification of the carboxyl group and the trifluoroacetyl radical for acylation of the amino group.

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1 E. BAYER, K. H. REUTHER AND F. BORN, *Angew. Chem.*, 69 (1957) 640.
2 H. A. SAROFF, A. KARMEN AND J. W. HEALY, *J. Chromatog.*, 9 (1962) 122.
3 E. BAYER, in D. H. DESTY (Editor), *Gas Chromatography 1958*, Butterworths, London, 1958, p. 333.
4 H. A. SAROFF AND A. KARMEN, *Anal. Biochem.*, 1 (1960) 344.
5 F. WEYGAND, B. KOLB, A. PROX, M. A. TILAK AND I. TOMIDA, *Z. Physiol. Chem.*, 322 (1960) 38.
6 F. WEYGAND, B. KOLB AND P. Z. KIRCHNER, *Anal. Chem.*, 181 (1961) 396.
7 J. WAGNER AND G. Z. WINKLER, *Anal. Chem.*, 183 (1961) 1.
8 G. ZOMZELY, G. MARCO AND E. EMERY, *Abstr. Papers, Meeting Am. Chem. Soc.*, Chicago, 1961, p. 48c.
9 C. G. YOUNGS, *Anal. Chem.*, 31 (1959) 1019.
10 D. E. JOHNSON, S. J. SCOTT AND A. MEISTER, *Anal. Chem.*, 33 (1961) 669.
11 F. WEYGAND AND R. GEIGER, *Chem. Ber.*, 89 (1956) 647.
12 C. H. NICHOLLS, S. MAKISUMI AND H. A. SAROFF, *J. Chromatog.*, 11 (1963) 327.

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A one-piece all-glass injection block and gas chromatographic column for the microanalysis of halogenated hydrocarbons

Reports of high-temperature decomposition of halogenated hydrocarbons resolved by gas chromatography have implicated the metallic surfaces of the column tubing as the site and the cause. We have recently shown[1] that improved recovery of this class of compounds may be obtained by replacing the metallic columns with a quartz column. This modification, however, did not completely eliminate contact of the compound with the metal parts of the instrument; *i.e.*, the injection area was largely metallic.

An all-glass, one-piece, injection block and chromatographic column has been designed and applied to the Dohrmann Model 100 gas chromatograph (Dohrmann Co., San Carlos Calif., U.S.A.), as illustrated in Fig. 1. The system is one piece up to the connection with the quartz pyrolysis tube, thereby eliminating all contact of the compound with a metal surface. It has been our experience that the slightest pin-hole leak will cause a measurable loss of the sample. This one-piece design has removed this problem.

The column is filled with the chromatographic packing prior to sealing it to the system and it must be cut off for refilling. However, the column will be efficient for a large number of samples provided that an efficient "cleanup" procedure has been applied to the sample prior to chromatography. This is especially true when agricultu-

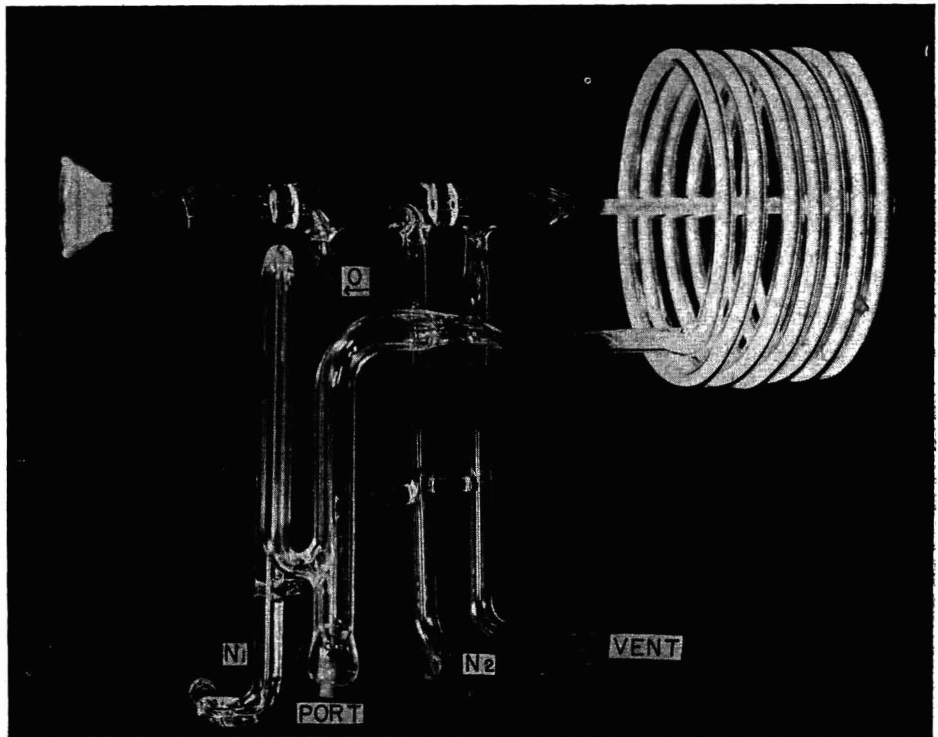

Fig. 1. All-glass, one-piece injection port and gas chromatographic column.

ral commodities are analyzed for trace amounts of halogenated organic contaminants.

The column packing was a portion of the same supply described previously, *i.e.*, 20% "purified" Dow-11 silicone oil on 40–50 mesh Chromosorb P. Therefore, the recovery data (Table I) may be directly compared with that previously reported[1].

TABLE I

GAS CHROMATOGRAPHY DATA ON HALOGENATED HYDROCARBONS USING AN ALL-GLASS SYSTEM

	Recovery (%)
p,p'-DDT (dichlorodiphenyltrichloroethane)	78
Technical DDT (isomeric mixture)	74
DDD (dichlorodiphenyldichloroethane)	82
DDE (dichlorodiphenyldichloroethylene)	89
Heptachlor (3,4,5,6,7,8,8-heptachlorodicyclopentadiene)	79
Heptachlor epoxide	95
Endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-endo-5,8-dimethano-naphthalene)	84
Dicloran (2,6-dichloro-4-nitroaniline)	98
2,4-DMe (2,4-dichlorophenoxyacetic acid methyl ester)	100

In Fig. 1, N_1 is the gas inlet that supplies the nitrogen carrier gas for the chromatography column; N_2 is the inlet for auxiliary nitrogen to propel the column effluents through the pyrolysis tube; O is the gas inlet that supplies oxygen to support combustion in the pyrolysis tube. The "vent", as marked in Fig. 1, allows the solvent vapors to be released to the outside through a valve, since it is unnecessary to combust the solvent. The vent valve is closed prior to the elution of the higher boiling halogenated compounds. The injection port is constructed of heavy-wall tubing to provide a sufficient reservoir of heat for complete vaporization of all materials injected into the system. Similarly, the tubing for the N_1 gas supply is of heavy-wall construction to provide preheated nitrogen to the injection chamber. Construction and directional jets are provided within the system to insure proper flow of gas streams throughout the system.

It has been noted that solvent vapors will ignite in the pyrolysis tube if they are not released through the vent port. This ignition can be observed as a flashback that permeates the entire system, back to the column including the oxygen and auxiliary nitrogen ports. Therefore, it is recommended that small volumes, 10 μl or less, be used and an ample vent period be allowed to avoid the flashback. This effect undoubtedly takes place in a metal system, but goes unobserved. Variable data could be ascribed to this effect.

Increased retention times (desirable with some compounds) and improved resolution of a mixture of some halogenated hydrocarbons have been observed if a large temperature differential can be maintained between the sample injection area and the chromatograph column, *i.e.*, high-temperature injection and a cooler column. This may be accomplished by inserting an asbestos-cement disc, 1/4-in. thick, between the injection and column areas of the all-glass system. The disc contains two slots in positions corresponding to the column spacing connection so that the disc may be easily inserted or removed without disturbing the system. The use of the asbestos disc

provides a temperature differential of as much as 100° between the sample injection and column areas.

If necessary, the injection port can be designed to include a removable pyrex or quartz tube insert which may be periodically removed for cleaning because of the possible accumulation of undesirable condensation products obtained from samples of plant, animal, or soil origin.

The recovery data obtained with the all-glass system are similar to those obtained with a quartz column and a metal injection block. The continued inability to realize better recoveries from some of the compounds studied suggests that other factors are involved in addition to contact of the compound with metallic surfaces. It must also be pointed out that the all-glass system was fabricated from borosilicate glassware. This was done for ease of construction as compared to an all-quartz piece. Since quartz was previously found to allow improved recoveries, the same effect may be true for an all-quartz system.

A combination of conjectural possibilities contributing to low recoveries may include formation of elemental chlorine, hydrogen chloride, adsorption of the compound or its decomposition products on the glass surface and reactivity with the column packing support. The first two possibilities seem the most logical in view of the fact that the recoveries shown are in direct molar ratio of the chlorine content of the compounds studied assuming the loss of one or more chlorine atoms. The loss of hydrogen chloride would show the same approximate values. This thesis will be studied further.

If the apparent loss does in effect show these phenomena to be true, then the only further improvement in the system would be to change the column packing material in such a way as to avoid the decomposition. It shows that the all-glass one-piece system has accomplished its purpose of providing optimum conditions for the recovery of these high-boiling halogenated compounds by avoiding leaks.

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HERMAN BECKMAN
ARTHUR BEVENUE

1 H. BECKMAN AND A. BEVENUE, *J. Chromatog.*, 10 (1963) 231.

Received February 4th, 1963

Impurities in analytical reagent grade chemicals

The rate of progress in chemistry is sensitive to the purity of the chemicals used in research work. In spite of the fact that many chemicals are sold as analytical reagent grade, it is discouraging to find that so many chemists need to purify chemicals further in order to ensure that the effects they are studying are not due to some impurity rather than the compound they are investigating. In our laboratory a working manual[1], on the purification of supposedly pure inorganic chemicals, has evolved during the years mainly through the painstaking work of DR. GEORGE BIEDERMANN and his associates.

The following experience with organic chemicals serves as an illustration of how hazardous it is to trust the labels on the bottles. In a program dealing with the principles of amine extraction, a technique useful in the reprocessing of nuclear fuels, we studied the extraction of water and nitric acid into xylene[2]. In Fig. 1 the results for water are given for two different bottles (I and II) of analytical reagent grade xylene from a well-known company. From Fig. 1 it is seen that the two samples

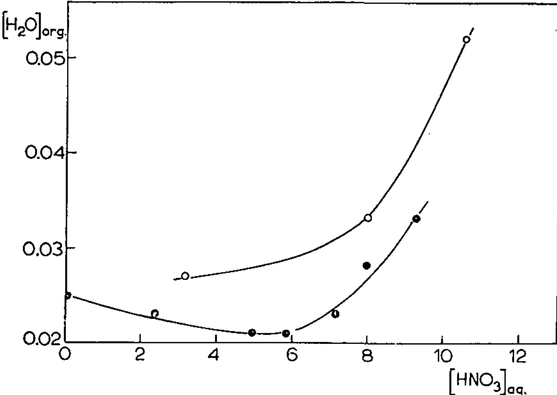

Fig. 1. The equilibrium molarity of water in the organic phase (determined by Karl Fischer titration) plotted against the equilibrium molarity of nitric acid in the aqueous phase for the system: xylene–HNO_3–H_2O. O = xylene I; ● = xylene II.

extract water quite differently. In order to check the purity of the two samples they were analysed by vapor phase chromatography (VPC). The results are shown in Table I, which shows that neither of the two samples meets with even very modest requirements of analytical purity. Samples as impure as these may be rare events;

TABLE I

VPC STUDY OF TWO DIFFERENT BATCHES OF XYLENE

Compound	*Constituent (%)*				
	m- + p-Xylene	*o-Xylene*	*Benzene*	*Toluene*	*Ethylbenzene*
Xylene I	89	6.7	0.6	0.4	3.2
Xylene II	59	21	—	1.1	18

unfortunately, however, it is not the first time the present writer has met with incredibly impure analytical reagent grade chemicals[3].

In order to prevent further experiences like these we now do a routine check on the purity of all volatile organic chemicals by VPC. However, not all research laboratories have access to a gas chromatograph. On the other hand the chemical industry can certainly afford to make routine checks by VPC in order to keep a uniform and high level of purity. Chemicals checked by VPC and labelled as gas chromatographically pure (to 99.5 % or better) would be of great help in scientific work. Although scientists constitute a small group of customers with strange and exclusive tastes, the feedback from research to industrial exploitation is of utmost economic importance. Guaranteed pure chemicals in the hands of the scientific workers would promote both speed and reliability in research work, to the advantage of all.

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E. HÖGFELDT

1 *Some Laboratory Methods* (*stencil*), Manual of the Department of Inorganic Chemistry, Royal Institute of Technology, Stockholm, July 1959.
2 E. HÖGFELDT AND B. BOLANDER, *Report No. 4 to AB Atomenergi*, November 1961.
3 E. HÖGFELDT AND J. BIGELEISEN, *J. Am. Chem. Soc.*, 82 (1960) 15.

Received January 14th, 1963

A sample applicator for column chromatography

When using column chromatography it is important to have an even packing and an even level column surface. The column surface must not be disturbed when the solution of compounds to be separated is applied, otherwise horizontal bands will not be produced.

The sample is usually dissolved in the minimum amount of eluent in a small beaker and applied to the top of the column by allowing it to run from a pipette rotated rapidly around the inner wall of the column. For quantitative work, the beaker must be rinsed several times with the minimum amount of eluent and the rinsings applied to the column as described. This can be a tedious process and great care must be taken not to disturb the column surface.

The column head described (made in glass) avoids disturbance of the packing and the necessity of transfer of a solution in quantitative work.

The reservoir (A) (Fig. 1) is connected through the tap (B) to a tube (C). The latter passes centrally through a standard cone (D) which fits into a standard socket (E) at the top of the column. A small length of soft plastic tubing (F) is attached to the end of the tube which is bent as shown. The free end of the plastic tubing is just in contact with the wall of the column. After lubrication of the joint, the column

head can be rotated manually by means of a rod (G) attached to the top of the cone (D). Access to the air is by a small hole (H).

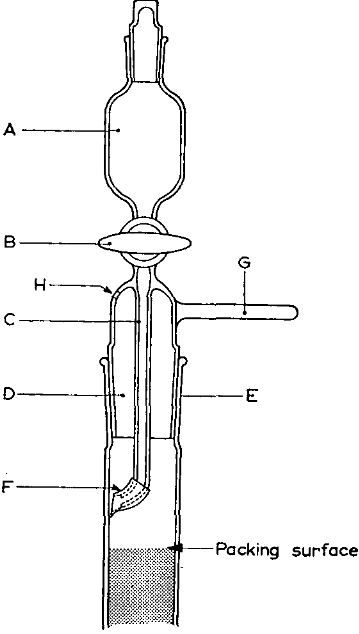

Fig. 1.

Procedure. The column head is fitted into the top of the column so that the end of the plastic tubing in contact with the wall is about $^1/_2$–1 inch above the packing surface. The solution is poured into the reservoir (A), the tap opened slightly and the column head rotated. The solution will then flow quickly down the sides of the column without disturbing the packing. Complete transfer can be effected by rinsing down the side of the reservoir and repeating the above procedure.

If the original mixture is a solid, it can be weighed in the column head and dissolved *in situ*, prior to application to the column.

Cotton Silk and Man-made Fibres Research Association,
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Manchester (Great Britain)

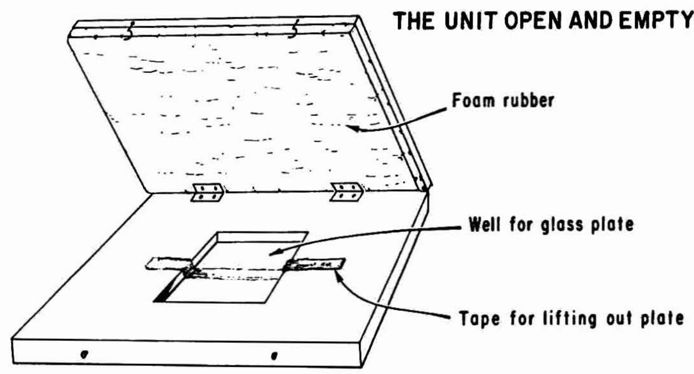
GEOFFREY A. BYRNE

Received February 7th, 1963

Radioautography of ^{14}C- and ^{3}H-labeled steroids on thin-layer chromatograms*

In order to extend the usefulness of thin-layer chromatography to experiments with trace amounts of isotopically labeled compounds of high specific activity it is necessary to detect the radioactivity on the plate. Radioautography permits the radioactivity to be recovered from one portion of the plate and the remainder to be sprayed with a suitable reagent to develop unlabeled reference compounds which have been chromatographed at the same time. Although the use of radioautography with thin-layer chromatograms has been reported[1], we believe the method described below is convenient and that our results will be usefull to other workers.

In order not to injure the coated surface of such a chromatogram during radioautography a special cassette was devised (Fig. 1). In this an 8 × 8 in. plate, coated side up, fits into a well of the same depth as the thickness of the glass. The fit is



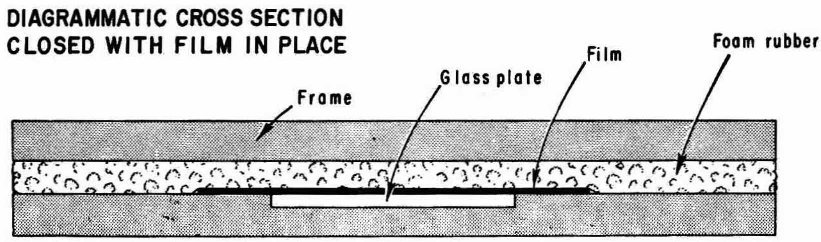


Fig. 1. Cassette for radioautography.

tight enough so that there can be no motion of the plate. (A strip of gauze laid under the plate so that the ends emerge on either side makes it easy to remove the plate from the well.) A layer of fine-texture foam rubber glued to the under surface of the lid of the cassette serves to hold the film against the plate in non-skid fashion with

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even pressure when the cassette is closed. This, plus a 3-in. margin in all directions around the plate, serves to exclude light.

An experiment with a ^{14}C-labeled compound was carried out and the resulting chromatogram is illustrated in Fig. 2. A solution of testosterone-4-^{14}C in 95 % aqueous

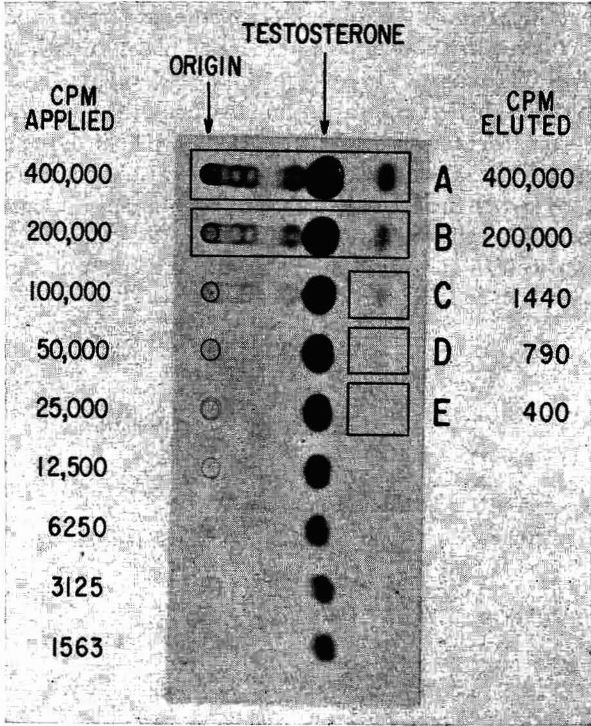

Fig. 2. Radioautograph of a chromatogram of a sample of testosterone-4-^{14}C.

ethanol containing 800,000 c.p.m./ml was prepared (1 μC equivalent to $1.1 \cdot 10^6$ c.p.m.). A thin layer (250 μ) of silica gel G (E. Merck, Darmstadt, Germany) on glass was prepared according to the method of STAHL[2]. A serial dilution of the testosterone solution was made and aliquots applied to the plate. The chromatogram was developed in ether–benzene (2:1, v/v). After evaporation of the solvent at room temperature Royal Blue X-ray film (Eastman Kodak, Co., Rochester, N.Y.) was placed in direct contact with the silica gel layer in the cassette.

At the end of 9 days the film was removed and developed in the prescribed manner. Radioactive areas on the plate were located by placing it over the film on a horizontal X-ray viewer. These areas were marked with a needle and then transferred from the plate into fluted filter papers. For this a nichrome spatula with one end ground to a straight, sharply beveled edge was used. The steroid was eluted with 20 ml of absolute methanol and aliquots of the eluates were counted. Areas A and B of Fig. 2 include the entire track as detected on the film. From each of these areas the same amount of material was recovered as had been applied at the origin, or 400,000 and 200,000 c.p.m. respectively. (In general we have been able to recover 90 %

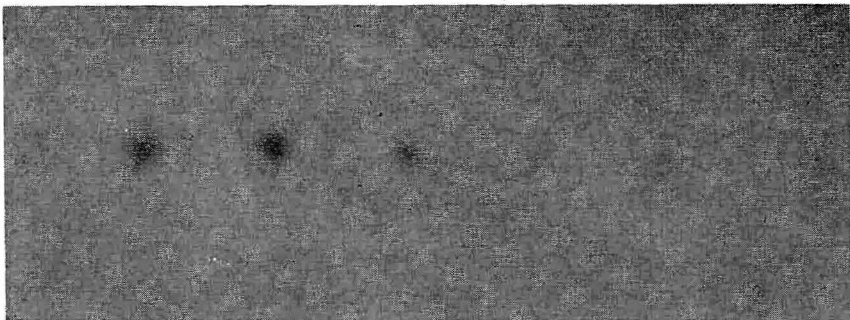

Fig. 3. Radioautograph of a chromatogram of 17α-hydroxypregnenolone-7α^{3}H.

or more of applied radioactivity.) Areas C, D and E, corresponding to the least exposed areas on the film, contained 1440, 790, and 400 c.p.m. respectively. The sites of application of material at the origin averaged 0.4 cm^2 in area and the less well defined areas of the spots at C, D and E averaged roughly 0.5 cm^2. The radioautograph, then, detects 400 c.p.m. ($4 \cdot 10^{-4}$ μC) of ^{14}C in 0.5 cm^2 in 9 days, and can be expected to detect 7200 c.p.m. ($7.2 \cdot 10^{-3}$ μC) per cm^2 in one day, or in more general terms $5 \cdot 10^6$ accumulated c.p.m./cm^2.

In order to make accurate recoveries of material from chromatograms having multiple radioactive and standard spots we have usually placed the chromatogram over a light source, covered it with a protective layer of cellophane, then placed the

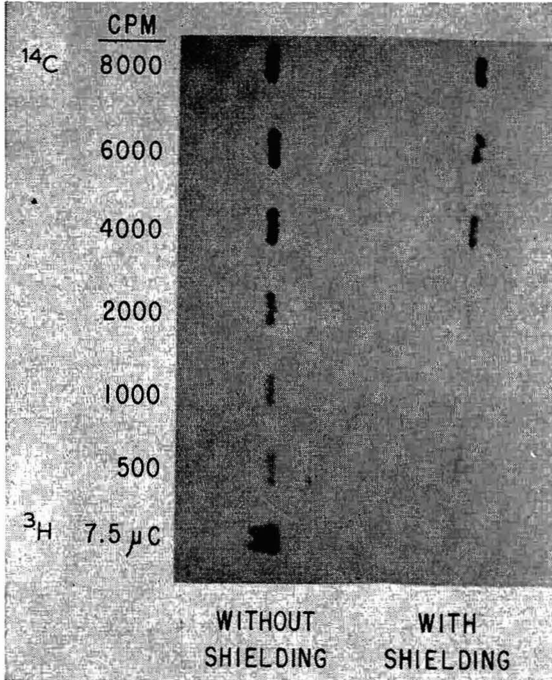

Fig. 4. Radioautograph of a chromatogram of steroids labeled with ^{14}C and tritium.

radioautograph over it, centering it by means of radioactive dye markers. If the exposed areas on the film are then traced with a pencil or similar instrument the outline is carried through to the plate without otherwise marking the surface. The areas so marked are removed, and the remainder of the chromatogram is sprayed to develop standards. The film is again placed on the chromatogram with a protective layer of cellophane in place and the outlines of the standard spots traced onto it in pencil. The X-ray film thus serves as the permanent record of the chromatogram.

The method applied to tritiated material is illustrated in Fig. 3 which shows a radioautograph of a chromatogram of 17α-hydroxypregnenolone-7α^{3}H. (New England Nuclear Corporation). From left to right 1.0 µC, 1.0 µC, 0.5 µC, 0.3 µC, and 0.2 µC, were applied at the origin. The chromatogram was run in ether–benzene (2:1, v/v) and radioautographed for 24 h.

The marked difference in energy level and range of ^{14}C and ^{3}H make it possible to differentiate between the two on a chromatogram by comparing a radioautograph with interposed shielding with another made without it. For ^{14}C the maximum energy is 0.156 meV and the range in aluminum is 25 mg/cm^2. For ^{3}H the corresponding figures[3] are 0.018 meV and 0.7 mg/cm^2. Approximately half of ^{14}C is adsorbed by 3 mg/cm^2 while ^{3}H is completely absorbed in such a layer. Fig. 4 shows a comparison of radioautographed spots containing the same amount of activity with and without shielding. In this instance Cellophane (Dupont C.), weighing 3.35 mg/cm^2., was used. It will be seen that the intensity of radiation as reflected by the density of the spot was reduced by about one-half in the case of ^{14}C, while in the case of ^{3}H only the faintest exposure of the film occurred on the shielded side even though 7.5 µC was present.

We have found that estradiol which is completely free of radioactivity as demonstrated by scintillation counting produces a faint autograph on X-ray film when present in amounts of 20 µg or more. While this is the only steroid thus far found to do this, it emphasizes the need for controls in radioautographing any new compound.

Vincent Memorial Laboratories of the Massachusetts General Hospital, Boston (Mass.); J. Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University and the Massachusetts General Hospital; and the Departments of Surgery and of Biological Chemistry, Harvard Medical School, Boston, Mass. (U.S.A.)

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W. BATCHELDER
M. GRIFFITH**
L. L. ENGEL***

1 H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.
2 E. STAHL, *Chemiker Ztg.*, 82 (1958) 323.
3 L. E. GLENDENIN, *Nucleonics*, 2 (1948) 12.

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*** Permanent Faculty Fellow of the American Cancer Society.

Book Reviews

Ion Exchange Separations in Analytical Chemistry, by O. SAMUELSON, Almquist & Wiksell, Stockholm, and John Wiley, New York and London, 1963, 474 pages, price Sw. Kr. 65.—.

The second edition of this well known monograph has been entirely rewritten. A general part deals with the properties of ion exchangers, ion exchange equilibria, kinetics, chromatographic theory and non-aqueous solutions. While some of these topics are described in the classic manner the reviewer found some sections, especially the chapter on non-aqueous solutions, very stimulating and full of new information.

A "practical part" surveys briefly the types of resins available and the techniques used with ion exchangers. "Applications" are divided into the use of ion exchangers in analytical procedures and in chromatographic separations. Both sections contain a wealth of data and full bibliographies which are remarkably up-to-date. The author has limited his field in various directions: he only describes work with synthetic ion exchange resins and just mentions inorganic ion exchangers; papers loaded with resins or other exchangers are also not treated and in the section on chromatographic separations only inorganic ions are dealt with. Thus a book containing all essentials for the analytical chemist has been created, while biochemical applications etc. must be sought elsewhere. In view of the very large literature on ion exchange separations the reviewer feels that this choice of topics was the best possible. The second edition will thus be just as indispensable for all analytical chemists as was the first during the last ten years.

MICHAEL LEDERER (Rome)

J. Chromatog., 12 (1963) 119

Chromatography and Electrophoresis on Paper. A Teaching Level Manual, by J. G. FEINBERG AND I. SMITH, Shandon Scientific Co., London, 1962, xii + 130 pages, 26 figs., 9 plates (colour), price £ 1.1s (25 % discount for educational establishments).

The speed at which these research techniques have reached the teaching syllabus of schools owes much to their inherent simplicity of execution, though problems of interpretation faced by researchers using the techniques may be other than simple. This simplicity of execution and the wide validity of the technique in dealing equally with substances of complex structure, such as chlorophyll, or with the simple inorganic ion, coupled with the visualisation step, makes these elegant research tools eminently attractive for catching the pupil's imagination and furthering the cause of scientific education.

J. Chromatog., 12 (1963) 119–120

The authors have succeeded in presenting a unified teaching system suitable for both University, College and sixth-form instruction, and with selection, for younger members of the school. The directions given are clear and the diagrams suitable. The colour plates are adequate but the hand printing on them rather detracts aesthetically. The index seems thorough and the book list for more advanced reading is useful, though not quite as balanced as it might have been. The historical chapters, in contrast, are well balanced as is the book as a whole. The choice of mixtures for separation, quite rightly, favours dyes and indicators though fruit juice and serum proteins are included for use in the more advanced exercises. As one might expect, with but two authors, the book has a welcome consistency of style not possible with multiple authorship such as that in Dr. SMITH's well known advanced book on the subject. One textual error stands out: the Norwegian FLOOD and the German LIESEGANG have been converted into Americans (p. 3). Some may find the description of partition and adsorption factors in mechanical terms debatable, whilst the substitution of *solubility* for *partition* seems an unnecessary "simplification" (pp. 8, 9).

The book's usefulness is partly limited by being tied to the publisher's equipment, used throughout the book. However, this limitation is probably less than it seems at first sight and it may even be a distinct advantage for teaching purposes.

The book and equipment are probably the first of a type designed specifically for instruction and they will, no doubt, make some effective contribution to scientific education. The authors and their colleagues are to be complimented on their initiative.

C. B. COULSON (Inveresk, Scotland)

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News

INTERNATIONAL SYMPOSIUM ON THIN-LAYER CHROMATOGRAPHY

The symposium was held at the Istituto Superiore di Sanità in Rome, on the 2nd and 3rd of May 1963, and there were over 200 participants.

The 8 lectures and numerous papers which were presented will be published in a special volume.

In addition to these communications, which are listed below, Dr. H. SEILER (Basel) showed a film on the technique of thin-layer chromatography with some inorganic separations.

Lectures

E. STAHL, Saarbruecken (Deutschland) — Development and application of thin-layer chromatography.

P. WOLLENWEBER, Dueren (Deutschland) — Dünnschicht-Chromatographie auf Cellulose-Schichten.

L. LABLER, Praha (Czechoslovakia) — Thin-layer chromatography on alumina.

E. DEMOLE, Genève (Suisse) — La chromatographie sur couches minces dans le domaine des substances odorantes naturelles et synthétiques.
G. GRASSINI, Roma (Italia) — Elettroforesi su strato sottile.
M. FÉTIZON, Gif-sur-Yvette (France) — Some applications of preparative thin-layer chromatography.
R. NEHER, Basel (Schweiz) — Thin-layer chromatography of steroids.
F. B. PADLEY, St. Andrews (Scotland) — Thin-layer chromatography of lipids.

Original papers

Techniques

M. BRENNER, Basel (Schweiz) — Standardised chromatographic data: a suggestion.
J. DAVIDEK, Prague (Czechoslovakia) — Chromatography on a thin-layer of starch with reversed phases.
J. ROSMUS, Prague (Czechoslovakia) — Centrifugal thin-layer chromatography.
M. VON SCHANTZ, Helsingfors (Finnland) (read by title only) — Über die Anwendung der Zirkulartechnik beim Chromatographieren auf Kieselgel-Dünnschichten. Trennung und Reindarstellung von Morphin, Papaverin und Chinin aus deren Gemischen.

Organic substances

H. J. PETROWITZ, Berlin (Deutschland) — Zur Dünnschichtchromatographie mehrkerniger aromatischer Kohlenwasserstoffe.
A. D'AMBROSIO, Milano (Italia) — Applicazione della cromatografia su strato sottile al riconoscimento e dosaggio degli idrocarburi.
G. M. NANO, Torino (Italia) — Thin-layer chromatography of 2,4-dinitrophenylhydrazones of aliphatic carbonyl compounds and their quantitative determination.
L. LABLER, Praha (Czechoslovakia) — Thin-layer chromatography of steroidal bases and *Holarrhena* alkaloids.
E. RAGAZZI, Padova (Italia) — Thin-layer chromatography of alkaloids on magnesia chromatoplates.
G. C. CASINOVI, Roma (Italia) — Alcune applicazioni della cromatografia su strato sottile per la separazione degli alcaloidi.
I. PEJKOVIC-TADIC, Belgrade (Jugoslavia) — Thin-layer chromatography of isomeric oximes. II.
J. OPIENSKA-BLAUTH, Lublin (Poland) — The adaptation of the technique of thin-layer chromatography to aminoaciduria investigation.
G. CURRI, Padova (Italia) — Direct analysis of phospholipids of mitochondria and tissue sections by thin-layer chromatography.
G. CAVINA, Roma (Italia) — Analisi qualitativa e quantitativa mediante cromatografia su strato sottile di corticosteroidi naturali e di sintesi.
C. BONINO, Bologna (Italia) — Thin-layer chromatography and the detection of stilboestrol.
J. W. COPIUS PEEREBOOM, Leiden (Netherlands) — Reversed phase thin-layer chromatography of sterol acetates.
P. FASELLA, Roma (Italia) — Thin-layer electrochromatography on sephadex and cellulose for the finger printing of proteins.
M. A. CIASCA, Roma (Italia) — Cromatografia di coloranti alimentari su strato sottile di gel di silice: dati preliminari.
M. COVELLO, Napoli (Italia) — The application of thin-layer chromatography to the research of antifermentatives in foodstuffs.

Inorganic substances

H. SEILER, Basel (Schweiz) — Direct quantitative determination of cations on thin layers.
G. BOTTURA, Bologna (Italia) — Techniche e principi della cromatografia su strato sottile di ioni metallici: schema di analisi di prodotti di fissione.
M. HRANISAVLJEVIC-JAKOVLJEVIC, Belgrade (Jugoslavia) — Thin-layer chromatography of inorganic cations. I. Separation of metal dithizonates.

Announcements

Volume 1 of the lectures given at the Postgraduate Summer School held at the Villa Farnesina in Rome from Sept. 17th–27th, 1962, has just appeared.

It contains 260 pages with numerous figures and tables. Price $ 12.00 post free.

It may be ordered directly from the Consiglio Nazionale delle Ricerche—Ufficio Pubblicazioni, Piazzale delle Scienze, 7, Roma, Italy or through any bookseller.

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E. CERRAI — Scambiatori ionici liquidi: separazioni su supporti inerti impregnati di scambiatori ionici liquidi (Liquid ion exchangers: separation on inert supports holding liquid ion exchangers). In Italian.
L. SACCONI — Cromatografia di adsorbimento nella chimica inorganica (Adsorption chromatography in inorganic chemistry). In Italian.
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G. ALBERTI — Separazioni cromatografiche su carte impregnate con scambiatori inorganici di sintesi (Chromatographic separation on paper impregnated with inorganic exchangers). In Italian.
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E. BLASIUS — Chromatographie und Komplexchemie: Ein Übersichtsreferat unter spezieller Berücksichtigung eigener Ergebnisse. In German.
J. P. EBEL — Chromatographie des phosphates condensés et autres polyanions. In French.
J. P. ADLOFF — Techniques et applications de la radiochromatographie en phase gazeuse. In French.

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GAS CHROMATOGRAPHY SYMPOSIUM

The Fifth International Symposium on Gas Chromatography to be organized by the Institute of Petroleum Gas Chromatography Discussion Group will be held on September 8, 9 and 10, 1964 at Brighton, England. The main aim of the symposium will be to launch a concentrated attack on the underlying physical processes responsible for separation. To achieve this, the morning sessions will be entirely devoted to this theme, the subjects for the three morning sessions being "Techniques of Separation and Identification", "Column Design", and "Exploitation of Molecular Interactions". Each morning session will be commenced by two papers given by recognized authorities. Informal discussions will be held during the afternoon sessions on a wide range of subjects relevant to the development and application of the technique. As at previous symposia, the technical programme will be accompanied by a full social programme and an instrument exhibition. Intending delegates and those wishing to submit papers should write to:

The Organizing Office,
Fifth International Symposium on Gas Chromatography,
61 New Cavendish Street, London, W.1.

The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy

EXPOSITION OF MODERN LABORATORY EQUIPMENT

Preliminary Announcement of 1964 Pittsburgh Conference

The Fifteenth Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy will be held at the Penn-Sheraton Hotel in Pittsburgh, Pa., U.S.A., March 2–6, 1964. Approximately 200 papers on all phases of analytical chemistry and spectroscopy will be presented. Symposia on the following subjects are proposed for the 1964 Conference :

1. Coblentz Society Symposium
2. Excitation Techniques
3. Electron Probe
4. Analysis of Polymers
5. Analysis of Gases in Metal including Activation Methods
6. Far Infrared
7. Adsorb Molecules

Original papers on all phases of analytical chemistry and spectroscopy are invited. A brief abstract (150 words) of each paper will be printed in the program. Three copies of this abstract, with a letter listing the names of the authors, the laboratory in which the work was done, and the current addresses of the authors, should be addressed to:

Mr. Rudolph B. Fricioni, Program Chairman
The Fifteenth Pittsburgh Conference
Allegheny Ludlum Steel Corporation
Corporate Quality Control Laboratory
Research Center
Brackenridge, Pa., U.S.A.

The final data for receipt of abstracts is October 15, 1963.

In addition to the program of technical papers, there will be an exhibition of the newest analytical instrumentation. More than 125 companies will display instruments, chemicals, and equipment. A complete program of activities for wives and lady attendees at the Conference is also being planned for the 1964 Pittsburgh Conference. Finally, the fifth OCEANS (Omnibus Conference on Experimental Aspects of NMR Spectroscopy) will be held at Mellon Institute, Pittsburgh, from Thursday, February 27, through Saturday, February 29, the week preceding this Conference. The first day will be devoted primarily to broad-line work, the second to experimental developments of general interest and the third to high resolution techniques. For the convenience of those wishing to attend both meetings, the NMR sessions of the Pittsburgh Conference will be scheduled for Monday, March 2.

Bibliography Section

EDITORIAL

From the inception of the publication of the bibliography of paper chromatography in May 1962, we were already convinced that similar bibliographies of other branches of chromatography would soon have to follow, given the general scope of the other sections of the *Journal of Chromatography*. Our group of abstractors has now been reorganised so that we can also present from now on bibliographies of THIN-LAYER CHROMATOGRAPHY and GAS CHROMATOGRAPHY in addition to PAPER CHROMATOGRAPHY. The present trend in research is such that most interest is centred on these three techniques. The number of papers on gas chromatography is almost as large as that on paper chromatography and the necessity for compiling a bibliography is beyond discussion (see also the Introduction by Ing. J. JANÁK, on p. 134).

Thin-layer chromatography, though already described long before paper chromatography, has only become popular during the last few years. It may be expected that its diffusion will increase rapidly since this method is highly suitable for the utilisation of the results and techniques of paper chromatography. The number of papers published is not yet equal to paper chromatography but is increasing logarithmically.

We intend to adhere to a uniform pattern in the Bibliography Section and hence will subdivide all three sub-sections into identical chapters. The subdivision of Chapters 2, 3, 4, 5 and 33 will be changed and the various chapters will now have the following titles:

1. Reviews and Books
2. Fundamentals, Theory and General
3. Techniques I
4. Techniques II
5. Hydrocarbons and Halogen Derivatives
6. Alcohols
7. Phenols
8. Substances Containing Heterocyclic Oxygen
9. Oxo Compounds
10. Carbohydrates
11. Organic Acids and Simple Lipids
12. Organic Peroxides
13. Steroids
14. Steroid Glycosides
15. Terpene Derivatives
16. Nitro and Nitroso Compounds
17. Amines, Amides and Related Nitrogen Compounds
18. Amino Acids
19. Peptides; Chemical Structure of Proteins and Peptides
20. Proteins
21. Purines, Pyrimidines, Nucleosides, Nucleotides, Nucleic Acids, Barbiturates
22. Alkaloids
23. Other Substances Containing Heterocyclic Nitrogen
24. Organic Sulphur Compounds
25. Organic Phosphorus Compounds

26. Metallo-organic Compounds
27. Vitamins
28. Antibiotics
29. Insecticides and Other Pesticides
30. Synthetic and Natural Dyes
31. Plastics and Their Intermediates
32. Pharmaceutical Applications
33. Inorganic Substances
34. Radioactive Compounds
35. Miscellaneou Compounds and Complex Mixtures

The general principles of the bibliography remain unchanged (see *J. Chromatog.*, 8 (1962) 138). In the thin-layer and gas chromatography bibliographies only papers published after January 1st, 1963, will be mentioned.

The thin-layer bibliography wil be compiled by the same team as the section on paper chromatography, while the bibliography of gas chromatography will be compiled by Ing. JAROSLAV JANÁK (Laboratory for Gas Analysis, Czechoslovak Academy of Sciences, Brno, Czechoslovakia) and his collaborators.

I would like to repeat my request to authors who publish in the less commonly read journals (which we only see through abstracting journals) to send reprints to:

Dr. KAREL MACEK
Research Institute for Pharmacy and Biochemistry,
Prague 3, Kouřimská 17, Czechoslovakia

so that we can mention their work with a minimum delay.

KAREL MACEK

Paper Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

GREEN, J. AND MARCINKIEWICZ, S.: Paper chromatography and chemical structure. I. Tankless or flat-bed chromatography. A method for the accurate determination of R_M values. *J. Chromatog.*, 10 (1963) 35–41.

MARCINKIEWICZ, S., GREEN, J. AND MCHALE, D.: Paper chromatography and chemical structure. The chromatography of phenols, alkoxyphenols, coumaranols and chromanols. The use of group and atomic R_M values. Steric and electronic effects in chromatography. *J. Chromatog.*, 10 (1963) 42–67.

THOMA, J. A.: Application and theory of unidimensional multiple chromatography. *Anal. Chem.*, 35 (1963) 214–224.

3. TECHNIQUES I

ARIMURA, A.: Glass paper chromatography. *Saishin Igaku*, 17 (1962) 1345–1354; *C.A.*, 57 (1962) 8830f.

BIDWELL, R. G. S.: Direct paper chromatography of soluble compounds in small samples of tissue adhering to the paper. *Can. J. Biochem. Physiol.*, 40 (1962) 757–761; *C.A.*, 57 (1962) 8829f — application of samples of tissue to paper.

COLEMAN, CH. H.: Solvent-front indicator for paper chromatograms. *U.S., Pat.*, 3,046,779, July 31, 1962, Appl. April 25, 1961, 3pp.; *C.A.*, 57 (1962) 13189b.

DESAGA, C., G.m.b.H., Nachf. ERICH FECHT (by H. WEIHRAUCH): (Developing chamber for two-dimensional paper chromatograms.) *Ger. Pat.*, 1,135,210 (Cl. 42), Aug. 23, 1962, Appl. Nov. 3, 1956, 4 pp.; *C.A.*, 57 (1962) 15812b.

KEMBER, N. F. AND FARMER, A.: The separation of metals on ion-exchange modified cellulose paper using complexing agents. *J. Chromatog.*, 10 (1963) 106–107.

LANINI, S. AND GAEUMANN, A.: (Quantitative evaluation of paper chromatograms by photometric reflectance measurement. The effect of the size of the spots.) *Schweiz. Arch. Angew. Wiss. Tech.*, 28 (1962) 267–268; *C.A.*, 57 (1962) 14409f.

ZAALISHVILI, M. M. AND SHRAÏBMAN, F. O.: (Universal apparatus for automatic application of spots to chromatographic paper.) *Biokhimiya*, 28 (1963) 9–12.

4. TECHNIQUES II

Systematic analysis, automation and preparative-scale paper chromatography

BEHRENS, U., RINGPFEIL, M. AND STRIEGLER, G.: (Identifying unknown materials by paper chromatography.) *Monatsber. Deut. Akad. Wiss. Berlin*, 3, No. 11–12 (1961) 635–638; *C.A.*, 57 (1962) 14409g.

IMPERIAL CHEMICAL INDUSTRIES OF AUSTRALIA AND NEW ZEALAND LTD. (by D. GUNEW): Paper chromatography for large scale separations. *Australian Pat.*, 233,713, May 5, 1961, Appl. April 20, 1959; *C.A.*, 57 (1962) 14911h.

7. PHENOLS

BENKO, F.: (Paper chromatography of "solutio fuchsini cum resorcino".) *Gyogyszereszet*, 6, No. 5 (1962) 177–178; *C.A.*, 57 (1962) 13890b.

CRUMP, G. B.: The paper chromatographic separation and identification of simple phenols. *J. Chromatog.*, 10 (1963) 21–28 — *o*- or *p*-nitrophenylazo dyes of 13 phenols.

KIELCZEWSKI, W. AND MILOSZEWSKA-PODOLAK, I.: (Determination of micro-amounts of pyrocatechol by an impregnation method on the filter paper.) *Chem. Anal.* (*Warsaw*), 8 (1963) 95–98.

9. OXO COMPOUNDS

HENNENBERG, M. AND OLSZAK, M.: (Chromatography of plant anthraquinone derivatives. II. Detailed part.) *Biul. Inst. Roslin Leczniczych*, 7 (1961) 255–263; *C.A.*, 57 (1962) 6245i — a review with 20 references.

HEYNS, K., GOTTSCHALK, H. AND PAULSEN, H.: Katalytische Oxydation der Kondurite (1,2,3,4-Tetrahydroxy-cyklohexene) zu Dehydro-konduriten (1-Oxo-2,3,4-trihydroxy-cyklohexene). *Chem. Ber.*, 95 (1962) 2660–2668 — R_F values of 10 compounds.

TÄUFEL, K. AND HEDER, G.: Zur Carbonylbildung bei autoxydierten Fettsäureestern. *Fette, Seifen, Anstrichmittel*, 65 (1963) 85–91.

10. CARBOHYDRATES

HOHNJEC-MIHALJINAC, S. AND BENZINGER, F.: (Saponins and other substances in the subterranean portions of *Primula vulgaris*. III. Sugar components of the isolated saponin.) *Sci. Pharm.*, 30 (1962) 46–47; *C.A.*, 57 (1962) 12633b.

MONTREUIL, J.: (Paper chromatography of sugars in blood; application to the study of galactosemias.) *Pathol. Biol. Semaine Hop.*, 10 (1962) 891–893; *C.A.*, 57 (1962) 6248g.

PECHAN, Z.: Paper chromatographic behaviour of low molecular weight hexosamine-containing compounds from human urine. *J. Chromatog.*, 10 (1963) 104–105.

STOLL, S. AND PRAT, Y.: (Detection and characterization of organic thickening agents by chromatography and electrorheophoresis.) *Ann. Fals. Expert. Chim.*, 55 (1962) 159–176; *C.A.*, 57 (1962) 16948d.

WALDI, D. AND LANGE, H. R.: Papierchromatographischer Nachweis von Dihydroxyaceton neben Glyzerin. *Naturwissenschaften*, 50 (1963) 126.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BAKER, B. R., LEE, W. W., MARTINEZ, A. P., ROSS, L. O. AND GOODMAN, L.: Potential anticancer agents. LXXVIII. Nonclassical antimetabolites. IV. Synthesis of compounds related to 4-(iodoacetamido)-salicylic acid, an *exo*-alkylating irreversible inhibitor. *J. Org. Chem.*, 27 (1962) 3283–3295 — R_F values of 10 compounds.

BOHM, B. A. AND TOWERS, G. H. N.: Phenolic compounds in *Impatiens*. *Can. J. Botany*, 40 (1962) 677–683; *C.A.*, 57 (1962) 6318c.

CERBULIS, J. AND TAYLOR, M. W.: Pyridine as a reagent for detection of acids on paper chromatograms. *Anal. Chem.*, 35 (1963) 114.

DUBOVSKÝ, J. AND PETRÁSEK, J.: (Phenolic acids in urine. II. Method for determination.) *Sb. Lekar.*, 64 (1962) 75–79; *C.A.*, 57 (1962) 6473a.

FISCHER, W. AND JAEHN, L.: (Paper chromatographic investigation of leather greases.) *Leder*, 13 (1962) 188–193; *C.A.*, 57 (1962) 16811f.

FREUDENBERG, K., CHEN, CHEN-LOUNG AND CARDINALE, G.: Die Oxydation des methylierten natürlichen und künstlichen Lignins. *Chem. Ber.*, 95 (1962) 2814–2828 — R_F values of 26 acids.

INOUE, Y.: Metabolism of *Streptomyces griseus*. V. Paper chromatographic study on organic and amino acids as metabolic products of *S. griseus*. *J. Antibiotics (Tokyo)*, A 11 (1958) 116–119; *C.A.*, 57 (1962) 7727b.

ISKHAKOV, N. I. AND SADYKOV, A. S.: (Fatty acid composition of cotton-seed oil.) *Dokl. Akad. Nauk Uz. SSR*, No. 11 (1961) 21–23; *C.A.*, 57 (1962) 6318h.

KOMODA, T. AND TAKESHITA, R.: Paper chromatography and paper electrophoresis of food preservatives. *Shokuhin Eiseigaku Zasshi*, 2 (1961) 72–76; *C.A.*, 57 (1962) 6369h.

KWAPNIEWSKI, Z. AND SLIWIOK, J.: (Note on the detection of saturated and unsaturated fatty acids on paper chromatograms.) *Nahrung*, 6, No. 4 (1962) 410; *C.A.*, 57 (1962) 12651g.

MUROOKA, H., KOBAYASHI, Y. AND ASAI, T.: Oxidative fermentation. XX. Catabolism of 5-ketogluconate by *Acetobacter liquefaciens*. *Agr. Biol. Chem. (Tokyo)*, 26 (1962) 135–141; *C.A.*, 57 (1962) 7718e.

NIKOLINSKI, P., BOYADZHIEVA, T., MLADENOV, I. AND SHIPOLINI R.: (Determination of some organic acids in rubber vulcanizates by paper chromatography.) *Godishnik Khim.-Tekhncl. Inst.*, 7, No. 1–2 (1960) 37–50; *C.A.*, 57 (1962) 13941g.

PAQUOT, C.: (Some new techniques used in fat analysis.) *Bull. Assoc. Franc. Chimistes Ind. Cuir Doc. Sci. Tech. Ind. Cuir*, 24 (1962) 221–230; *C.A.*, 57 (1962) 13903b.

13. STEROIDS

GOSZTONYI, T., MÁRTON, J., KEMÉNYI, V. AND VECSEI (WEISZ), P.: Reactions of corticosteroids with tetrazolium salts on paper chromatograms. *J. Chromatog.*, 10 (1963) 29–34.

HÄHNEL, R.: Isolation of oestriol-16(17?)-glucosiduronate by means of an ion exchange resin. *Clin. Chim. Acta*, 8 (1963) 305–307.

PASQUALINI, J. R. AND JAYLE, M. F.: Identification des corticostéroïdes dans les urines d'un sujet atteint d'une tumeur féminisante des corticosurrénales. *Clin. Chim. Acta*, 8 (1963) 283–289 — R_F values of 10 steroids.

SCIARRA, F., LUCHETI, L. AND DEFELICE, M.: (Partition paper chromatography and fluorometric evaluation in determination of urinary estrogens.) *Folia Endocrinol. (Pisa)*, 14 (1961) 694–704; *C.A.*, 57 (1962) 6535c.

14. STEROID GLYCOSIDES

BOICHINOV, A. AND PANOVA, D.: (Saponins from *Polygala major*.) *Farmatsiya (Sofia)*, 12, No. 3 (1962) 19–26; *C.A.*, 57 (1962) 13887f.

GULYI, E. A. AND LOSHKAREV, P. M.: (Quantitative determination of lanatosides A, B, C and D in *Digitalis lanata*.) *Med. Prom. SSSR*, 16, No. 1 (1962) 41–45; *C.A.*, 57 (1962) 12636h.

HOHNJEC-MIHALJINAC, S. AND BENZINGER, F.: (Saponins and other substances in the subterranean portions of *Primula vulgaris*. I.) *Sci. Pharm.*, 30 (1962) 3–16; *C.A.*, 57 (1962) 12632i.

KOWALEWSKI, Z.: (Quantitative determination of strophantidin heterosides after chromatographic separation of extracts of seeds of *Erysimum perofskianum*.) *Biul. Inst. Roslin Leczniczych*, 7 (1961) 211–238; *C.A.*, 57 (1962) 6245h.

STUDER, P., PAVANARAM, S. K., GAVILANES, C. R., LINDE, H. AND MEYER, K.: Glykoside der Blätter von *Digitalis canariensis* L. 1. Mitteilung. *Helv. Chim. Acta*, 46 (1963) 23–45 — paper and thin-layer chromatography.

VOLPP, G. AND TAMM, Ch.: 1α, 11α-Oxido-strophanthidol (Monoanhydro-ouabagenin) sowie weitere 1α, 11α-Oxido-steroide aus Ouabagenin. *Helv. Chim. Acta*, 46 (1963) 219.

15. TERPENE DERIVATIVES

ATTAWAY, J. A., WOLFORD, R. W., ALBERDING, G. E. AND EDWARDS, G. J.: Paper chromatographic analysis of terpene alcohols as their *o*-nitrophenyl- and *p*-phenylazophenylurethan derivatives. *Anal. Chem.*, 35 (1963) 234–236 — R_F values of 23 terpene alcohols.

HÖRHAMMER, L., RICHTER, G. AND WAGNER, H.: Papierchromatographie von ätherischen Ölen. *J. Chromatog.*, 10 (1963) 108–110 — on paraformaldehyde-treated paper.

16. NITRO AND NITROSO COMPOUNDS

COPPI, G.: (Chromatographic separation and determination of the nitrobenzoic acid isomers.) *Boll. Chim. Farm.*, 101 (1962) 225–230; *C.A.*, 57 (1962) 14439f.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

CIURLO, R.: (Paper chromatographic identification and separation of *p*-aminosalicylic acid hydrazide, ethyl *p*-aminosalicylate, *p*-aminosalicylic acid (PAS), and *m*-aminophenol (MAP).) *Rass. Chim.*, 14, No. 3 (1962) 103–104; *C.A.*, 57 (1962) 16749i.

DE JONG, H. G. BUNGENBERG: (Chromatography of long-chain electrolytes and their complexes considered in relation to the chromatography of lecithin and cephalin.) *Koninkl. Ned. Akad. Wetensch., Proc.*, B 65 (1962) 144–153, 154–163; *C.A.*, 57 (1962) 6247h.

STERESCU, M. AND PELLONI, V.: (Identification and determination of *o*-phenylenediamine in residual waters, in the synthesis of 2-sulfanilamidoquinoxaline sulfaquinoxaline), by paper chromatography.) *Rev. Chim. (Bucharest)*, 12 (1961) 296; *C.A.*, 57 (1962) 13562b.

YOMOGIDA, S.: Detection of ethinamate and its metabolic product in urine. *Kagaku Keisatsu Kenkyusho Hokoku*, 15 (1962) 86–89: *C.A.*, 57 (1962) 6247e.

18. AMINO ACIDS

BERRY, H. K., SCHEEL, C. AND MARKS, J.: Microbiological test for leucine, valine, and isoleucine using a urine sample dried on filter paper. *Clin. Chem.*, 8 (1962) 242–245; *C.A.*, 57 (1962) 8839a.

BISERTE, G., HAN, K., PAYSANT, P. AND BOULANGER P.: Acides aminés et polypeptides des liquides biologiques. X. Étude qualitative et quantitative de liquides d'ascite d'étiologies diverses. *Clin. Chim. Acta*, 8 (1963) 359–375.

CEGIELKA, M.: (A qualitative study on the amino acids in the semen from ox, dog and pig.) *Nord. Veterinarmed.*, 10, No. 2 (1958) 93–101; *C.A.*, 57 (1962) 6472h.

CIRCO, R. AND FREEMAN, B. A.: Diethylamine–ninhydrin as a color test aid in identification of amino acids on paper chromatograms. *Anal. Chem.*, 35 (1963) 262–263.

FISCHL, J. AND SEGAL, S.: "Small volume" electrophoresis. II. Electro-chromatography. *Clin. Chim. Acta*, 8 (1963) 479–481.

IRREVERRE, F., MORITA, K., ISHII, S. AND WITKOP B.: Occurrence of *cis*- and *trans*-3-hydroxy-L-proline in acid hydrolyzates of telomycin. *Biochem. Biophys. Res. Commun.*, 9 (1962) 69–71; *C.A.*, 57 (1962) 16745g.

KHARAT'YAN, A. M.: (Application of ash-free paper for chromatography.) *Lab. Delo*, 7, No. 9 (1961) 8–11; *C.A.*, 57 (1962) 11835a.

LIN, JUNG-YAO AND LIN, KUO-HUANG: Free amino acids in the seeds of *Leucaena glauca*. I. Isolation and identification of mimosine. *T'ai-wan I Hsueh-Hui Tsa Chih*, 60 (1961) 651–656; *C.A.*, 57 (1962) 7577d.

LISSITSKY, S. AND BISMUTH, J.: Détermination quantitative de la composition du sérum en ^{131}I-thyroxine et ^{131}I-triiodothyronine par filtration sur gel de dextrane (Sephadex). *Clin. Chim. Acta*, 8 (1963) 269–276.

MEDVEDEVA, M. N., KUGENEV, P. V. AND SHCHUKA, V. P.: (Paper chromatographic method for serial determination of amino acids.) *Lab. Delo*, 7, No. 9 (1961) 3–8; *C.A.*, 57 (1962) 11832d.
PFISTERER, H. G.: (Amino acid chromatography after total gastrectomy.) *Protides Biol. Fluids, Proc. 9th Colloq., Bruges, Belg.*, (1961) 319–321: *C.A.*, 57 (1962) 7541d.
REVICZKY, A. L., SZÁNTÓ, L. AND FORGÁCS, P.: Die Frontsubstanz der Schilddrüsen-Papierchromatogramme. *Arzneimittel-Forsch.*, 13 (1963) 72–74.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

KOLOTILOVA, A. I., KOROVKIN, B. F., LYZLOVA, S. N., VAGNER, V. K., VASILENKO, E. T. AND DZUTSOV, N. K.: (Free ribonucleotides and activity of some enzymes of the pentose phosphate cycle in experimental myocard infarct.) *Biokhimiya*, 28 (1963) 113–121.
REIST, E. J., BENITEZ, A., GOODMAN, L., BAKER, B. R. AND LEE, W. W.: Potential anticancer agents. LXXVI. Synthesis of purine nucleosides of β-D-arabinofuranose. *J. Org. Chem.*, 27 (1962) 3274–3279 — R_F values of 7 nucleosides.
REIST, E. J., BENITEZ, A., LEE, W. W., BAKER B. R. AND GOODMAN, L.: Potential anticancer agents. LXXVII. Synthesis of nucleosides of purine-6-thiol (6-mercaptopurine) containing "Fraudulent" sugars. *J. Org. Chem.*, 27 (1962) 3279–3283.
STEVENS, H. M.: The resolution of Tuinal and other barbiturate mixtures. *Med., Sci. Law*, 2 (1962) 268–273; *C.A.*, 57 (1962) 13890i.
YAMAMURA, J. AND KATO, N.: (Paper chromatography of barbiturates with salt-impregnated papers.) *Kagaku Keisatsu Kenkyusho Hokoku*, 15 (1962) 223–225: *C.A.*, 57 (1962) 12635c.

22. ALKALOIDS

AGURELL, S. AND RAMSTAD, E.: Analysis of clavine alkaloids of *Pennisetum ergot. Lloydia*, 25 (1962) 67–77; *C.A.*, 57 (1962) 16746e.
DEBSKA, W.: (Separation of atropine, *l*-hyoscyamine, and their salts.) *Bull. Soc. Amis Sci. Lettres Poznan*, C 11 (1962) 97–102; *C.A.*, 57 (1962) 13886g.
GLOCK, E. AND WRIGHT, M. P.: Determination of nornicotine in tobacco and smoke by the 1,3-indanedione spectrophotometric method. Comparison with an improved paper chromatographic–ultraviolet spectrophotometric procedure. *Anal. Chem.*, 35 (1963) 246–251.
GURRIERI, S. AND MUSUMECI, S.: (Identification of cocaine in Novocaine by paper chromatography.) *Boll. Sedute Accad. Gioenia Sci. Nat. Catania*, 5 (1960) 489–496; *C.A.*, 57 (1962) 15242e.
IRMSCHER, H.: (Notes on rapid identification of hyoscyamine in belladonna plants by paper chromatography.) *Pharm. Praxis, Beilage Pharmazie*, (1961) 184; *C.A.*, 57 (1962) 13889c.
KOEN, V.: (Identification of atropine, dionine and papaverine in pharmaceutical preparations by paper chromatography.) *Farmatsiya (Sofia)*, 12, No. 3 (1962) 36–40; *C.A.*, 57 (1962) 12634h.
LUCKNER, M. AND MOTHES, K.: Zur Bildung von Chinolinalkaloiden in Pflanzen. 1. Die Biosynthese von Viridicatin. *Arch. Pharm.*, 296 (1963) 18–33.
MARTINEZ DE CAZON, E.: (Identification and separation of strychnine. Paper chromatography and microcrystallography toxicology.) *Publ. Inst. Invest. Microquim., Univ. Nacl. Litoral (Rosario, Arg.)*, 24, No. 22 (1958) 116–124; *C.A.*, 57 (1962) 6243d.
MATHIS, C. AND DUQUÉNOIS, P.: Contribution à l'identification par chromatographie sur papier des alcaloïdes de quelques strychnos d'Afrique et d'Asie. *Ann. Pharm. Franc.*, 21 (1963) 17–26.
POPA, M. AND STERESCU, M.: (Identification and determination of theophylline in the presence of glucose and sodium salicylate by paper chromatography.) *Rev. Chim. (Bucharest)*, 11 (1960) 420; *C.A.*, 57 (1962) 16752e.
POPELAK, A., LETTENBAUER, G. AND HAACK, E.: Über Halogen- und "Pseudohalogen-" Verbindungen des 18-Desoxy-methylreserpats. *Arch. Pharm.*, 296 (1963) 67–72 — R_F values of 14 reserpoids.
SZENDEY, G. L.: Die U.V.-Absorptionsuntersuchung einiger quartärer Tropeine mit Biphenylgruppe. *Arch. Pharm.*, 296 (1963) 121–126 — R_F values of 11 compounds.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

ERSPAMER, V. AND BERTACCINI, G.: The antidiuretic action and the fate of 5-hydroxy-DL-tryptophan in the rat. *Arch. Intern. Pharmacodyn.*, 137 (1962) 6–23; *C.A.*, 57 (1962) 7840d.
KARTOZIYA, Ts. I.: (The Van den Bergh reaction and the chromatography of bilirubin.) *Soobshch. Akad. Nauk. Gruz. SSR*, 28 (1962) 41–46; *C.A.*, 57 (1962) 8830f.
LIST, P. H. AND WAGNER, K.: Ustilagin und andere Inhaltsstoffe des Maisbrander, *Ustilago maydis* Corda. *Arzneimittel-Forsch.*, 13 (1963) 36–41 — R_F values of 15 imidazole and guanidine derivatives.

SCHENK, W.: Colorimetrische Bestimmung der 4-Hydroxypipecolinsäure, 4-Aminopipecolinsäure und Pipecolinsäure mit Ninhydrin in Pflanzenextrakten. *Biochem. Z.*, 336 (1962) 557–563.

WAKSMUNDZKI, A., SOCZEWINSKI, E. AND RATAJEWICZ, D.: (The chromatographic separation of some acridine derivatives.) *Chem. Anal.* (*Warsaw*), 8 (1963) 103-106.

24. ORGANIC SULPHUR COMPOUNDS

SAMPAOLO, A.: The metabolites of O,O-dimethyldithiophosphorylacetic acid N-monomethylamide (active principle of Rogor) in olive oil. *Rend. Ist. Super. Sanita*, 24 (1961) 936-949; *C.A.* 57 (1962) 7681d.

SHESTOV, A. P. AND OSIPOVA, N. A.: (Sulfonation of aromatic compounds.) *Sb. Statei, Nauchn.-Issled. Inst. Organ. Poluprod. i Krasitelei*, No. 2 (1961) 13–45; *C.A.*, 57 (1962) 16511d.

YAMAMOTO, N., SHIMAMOTO, I. AND KOBAYASHI, M.: (Paper chromatography of anionic surfactants.) *Kogyo Kagaku Zasshi*, 62 (1959) 1549–1551; *C.A.*, 57 (1962) 15265a.

25. ORGANIC PHOSPHORUS COMPOUNDS

BRIDGES, R. G., CRONE, H. G. AND BEARD, J. R.: A study of the phospholipids of dieldrin-resistant and susceptible house flies, with particular reference to those of the thoracic ganglion. *Radioisotopes Radiation Entomol., Proc. Symp., Bombay, India* (1960, Publ. 1962) 145–153; *C.A.*, 57 (1962) 7754c.

27. VITAMINS

BOECKEL, C. R. C.: (Paper chromatography of vitamins B_1, B_2 and nicotinic acid.) *Semana Med.* (*Buenos Aires*), 120 (1962) 344–345; *C.A.*, 57 (1962) 12639d.

GAY, S.: (Properties of hydroxocobalamin.) *Biochim. Appl.*, 9 (1962) 65–70; *C.A.*, 57 (1962) 13889b.

JAENICKE, L. AND KOCH, J.: Zum Mechanismus der Carboligasereaktion. Hydroxymethyl-thiaminpyrophosphat, ein neues aktiver Kohlenstofffragment. *Biochem. Z.*, 336 (1962) 432–443.

NAGAWA, M., KATAOKA, M. AND YOSHIOKA, T.: Allied compounds of vitamin B_1. XXV. Synthesis of S-acylthiamine O-monophosphates. *Takamine Kenkyusho Nempo*, 13 (1961) 20–23; *C.A.*, 57 (1962) 15087b.

28. ANTIBIOTICS

SFERRUZZA, G. AND RANGONE, R.: (Chromatographic determination of novobiocin in presence of its isomerization and degradation products.) *Farmaco* (*Pavia*), *Ed. Prat.*, 17 (1962) 404–415; *C.A.*, 57 (1962) 16749d.

30. SYNTHETIC AND NATURAL DYES

HERNDON, J. F., TOUCHSTONE, J. C., WHITE, G. R. AND DAVIS, C. N.: Horizontal chromatography accelerating apparatus. Separation of dyes and indicators. *Anal. Chem.*, 35 (1963) 238–240 — R_F values of 22 dyes.

MORVAY, J.: (Circular paper chromatography for the modification of "foreign dye" test (Hungarian Pharmacopeia V) and for the identification of "solutio tricolorata".) *Acta Pharm. Hung.*, 28, No. 5–6 (1958) 200–206; *C.A.*, 57 (1962) 12636b.

OSTERLOH, F.: Das Verhalten von Farbstoffen mit Thiosulfat-Gruppen beim Färben von Wolle. *Melliand Textilber.*, 44 (1963) 57–62.

PLA-DELFINA, J. M.: Systematic identification of food, drug and cosmetic azo dyes. *J. Soc. Cosmetic Chemists*, 13 (1962) 214–244; *C.A.*, 57 (1962) 11861g.

PUCHE, R. C. T.: (Determination of synthetic food colors by paper chromatography.) *Publ. Inst. Microquim., Univ. Nacl. Litoral* (*Rosario, Arg.*), 24, No. 22 (1958) 176–184; *C.A.*, 57 (1962) 6368f.

REIF, J.: Die Papierchromatographie der Reaktivfarbstoffe. *Deut. Textiltech.*, 13 (1963) 86–89.

SADINI, V.: (Further developments on chromatographic research on the coloring matter of foods.) *Rass. Chim.*, 13 (1961) 13–18; *C.A.*, 57 (1962) 6368g — a review with 100 references.

SENN, R. CH., STAMM, O. A. AND ZOLLINGER, HCH.: Eigenschaften der Bindung zwischen Reaktivfarbstoffen und Zellulose. *Melliand Textilber.*, 44 (1963) 261–266.

ŠRÁMEK, J.: (Systematic analysis of textile dyestuffs by means of paper chromatography.) *Textil*, 18 (1963) 70–74.

ŠRÁMEK, J.: (Substantivity of reactive dyes.) *Textil*, 18 (1963) 190–191.

ŠRÁMEK, J.: (Application of paper chromatography to the analysis of textile materials.) *Textilnaya Prom.*, 23 (1963) 69–72.

VLASENOK, L. I.: (Chromatographic separation of chlorophyllide A, chlorophyllide B and protochlorophyllide on paper.) *Dokl. Akad. Nauk Belorussk. SSR*, 6 (1962) 255–259; *C.A.*, 57 (1962) 8830g.

VLASENOK, L. I. AND SHLYK, A. A.: (Chlorophyllide as an intermediate product in the transformation of protochlorophyllide into chlorophyll.) *Biokhimiya*, 28 (1963) 57–69.
WOIDICH, H.: (Analysis of red and black currant juices.) *Intern. Fruchtsaft-Union, Ber. Wiss.-Tech. Kom.*, No. 2 (1959) 69–74; *C.A.*, 57 (1962) 6378h.

31. PLASTICS AND THEIR INTERMEDIATES

ITO, Y.: Two-dimensional paper chromatography of urea–formaldehyde resin intermediates. *Kogyo Kagaku Zasshi*, 62 (1959) 1918–1920; *C.A.*, 57 (1962) 12702b.
WEIGEL, K.: (Basic paper chromatography of resins.) *Gummi, Asbest, Kunststoffe*, 15 (1962) 20, 24, 26, 28, 32, 34 and 36; *C.A.*, 57 (1962) 12712g.

32. PHARMACEUTICAL APPLICATIONS

DEQUEKER, R.: (Determination of the anthracene derivatives in senna leaves and tinctures.) *Boll. Chim. Farm.*, 101 (1962) 290–302; *C.A.*, 57 (1962) 12633i.
SZASZ, G., KHIN, L., TAKACS, M. AND SZASZ, M.: (Separation of drugs by paper chromatography.) *Acta Pharm. Hung.*, 28, No. 5–6 (1958) 219–228; *C.A.*, 57 (1962) 12638g.
VEGH, A., SZASZ, G. AND PAPP, O. B.: (Azophen purity determination. Paper chromatographic separation of 1-phenyl-3-methyl-5-pyrazolone and azophen.) *Acta Pharm. Hung.*, 29 (1959) 212–216; *C.A.*, 57 (1962) 12635a.

33. INORGANIC SUBSTANCES

BLASIUS, E. AND WERMBTER, G.: (Investigation of the Wackenroder reaction by means of radiopaper chromatography. IV.) *Z. Anorg. Allgem. Chem.*, 316 (1962) 251–260; *C.A.*, 57 (1962) 16121f.
CHOU, S.-F. AND LIANG, S.-CH.: Separation of vanadium, molybdenum, and tungsten by means of paper chromatography. II. *Sci. Sinica (Peking)*, 11, No. 2 (1962) 207–210; *C.A.*, 57 (1962) 11837a.
CONNOLLY, J. F. AND MAGUIRE, M. F.: An improved chromatographic method for determining trace elements in foodstuffs. *Analyst*, 88 (1963) 125–130 — quantitative analysis *in situ*.
GATTOW, G. AND SCHOTT, D.: R_F-Werte bei der Papierchromatographie von Wismuth. *Naturwissenschaften*, 50 (1963) 18.
HÁGONY, P. L. AND JÁKY, M.: Qualitative and quantitative Untersuchung kondensierter Phosphate. *Fette, Seifen, Anstrichmittel*, 65 (1963) 235–239.
IMAI, H.: The paper chromatographic separation of calcium, strontium and barium with an inorganic developer. *Nippon Kagaku Zasshi*, 82 (1961) 1176–1179; *C.A.*, 57 (1962) 13168i.
KAPLAN, B. YA. AND OLSHEVSKAYA, I. V.: (Estimation of scandium in the coal-ash after paper chromatographic resolution.) *Zavodsk. Lab.*, 29 (1963) 26–27.
KIELCZEWSKI, W. AND SUPINSKI, J.: (Determination of microgram amounts of cobalt by paper impregnation method.) *Chem. Anal. (Warsaw)*, 8 (1963) 59–62 — quantitative analysis *in situ* (visual).
LEDERER, M., MOSCATELLI, V. AND PADIGLIONE, C.: Chromatography on paper impregnated with ion-exchange resins. VII. Ion exchange with organic solvents. *J. Chromatog.*, 10 (1963) 82–92.
OCARANZA, E. R.: (Reaction of cobalt with hydantoins, chromatography on paper, interferences, sensitivity and applications to vitamin B_{12}, qualitative analysis, alloys and minerals.) *Publ. Inst. Invest. Microquim., Univ. Nacl. Litoral (Rosario, Arg.)*, 24, No. 22 (1958) 166–175; *C.A.*, 57 (1962) 15783a.
OLENOVICH, N. L. AND CH'I, W.-I.: (Separation of indium and cadmium by paper chromatography.) *Zh. Analit. Khim.*, 17 (1962) 522–523; *C.A.*, 57 (1962) 14414f.
POLLARD, F. H., NICKLESS, G. AND WARRENDER, R. W.: Chromatographic studies on the hydrolysis of phosphorus compounds. V. The hydrolysis of hexametaphosphimic acid. *J. Chromatog.*, 10 (1963) 73–77.
POLLARD, F. H., NICKLESS, G. AND WARRENDER, R. W.: Chromatographic studies on the hydrolysis of phosphorus compounds. VI. The hydrolysis of sodium heptametaphosphimate and octametaphosphimate. *J. Chromatog.*, 10 (1963) 78–81.
SHERMA, J. A.: Separation of certain cations from mictures of various cations on ion-exchange papers. I. Silver and thallium. *Talanta*, 9 (1962) 775–780; *C.A.*, 57 (1962) 13168d.
SZARVAS, P., BALOGH-KELEMEN, I. AND JARABIN, Z.: (Rutin in chromatographic inorganic analysis.) *Acta Univ. Debrecen.*, 7, No. 2 (1961) 119–124; *C.A.*, 57 (1962) 14417a.

34. RADIOACTIVE COMPOUNDS

GHIGLIONE, C. AND DUMAZERT, Ch.: (Application of radioisotopic indicators to paper chromatography.) *Bull. Soc. Pharm. Marseille*, 11 (1962) 3–26; *C.A.*, 57 (1962) 16746b.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

LEWIN, S. Z.: VII. Chromatographic glossary. *J. Chem. Educ.*, 40 (1963) A 167 – A 184 — terminology of thin-layer and gas chromatography in German, English, French and Spanish.

WALDI, D.: Fortschritte in der Dünnschicht-Chromatographie. *Mitt. Deut. Pharm. Ges.*, 13 (1963) 1–8 — a review with 31 references.

3. TECHNIQUES I

BEKERSKY, I.: Spray technique for the preparation of thin layer chromatography plates. *Anal. Chem.*, 35 (1963) 261–262.

BOYD, G. S. AND HUTTON, H. R. B.: Detection of steroids on chromatoplates using a non-destructive method. *Biochim. Biophys. Acta*, 69 (1963) 419–420 — ultraviolet lamp (max. 254 mμ) and thin-layer with inorganic phosphors.

HANSBURY, E., OTT, D. G. AND PERRINGS, J. D.: Thin-layer chromatography. Apparatus. *J. Chem. Educ.*, 40 (1963) 31.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

KLOUBEK, J. AND ETTEL, V.: Über die Isolierung und Identifizierung von 1-Methyl-1-äthyl-2-phenyl-2-tolyläthylen aus dem Abfallprodukt bei der Raffination der Toluolfraktion. *Collection Czech. Chem. Commun.*, 28 (1963) 397–403.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

HÖRHAMMER, L., WAGNER, H. AND SALFNER, B.: Neue Flavonglykoside aus der Kamille (*Matricaria chamomilla* L.). *Arzneimittel-Forsch.*, 13 (1963) 33–36.

9. OXO COMPOUNDS

HENNINGER, M. D., DILLEY, R. A. AND CRANE, F. L.: Restoration of ferricyanide reduction in acetone-extracted chloroplasts by β- and γ-tocopherol quinones. *Biochem. Biophys. Res. Commun.*, 10 (1963) 237–242.

KLOUWEN, M. H., TER HEIDE, R. AND KOK, J. G. J.: Dünnschicht-Chromatographie. II. Trennung aromatischer Aldehyde. *Fette, Seifen, Anstrichmittel*, 65 (1963) 414–418.

TREBST, A. AND ECK, H.: Über eine *p*-Hydroxylierung in isolierten Chloroplasten. *Z. Naturforsch.*, 18b (1963) 105–110.

WESSELY, F., ZBIRAL, E. AND JÖRG, J.: Über die Einwirkung von Bleitetraacetat auf Phenole. 9. Mitt. Die Oxydation einiger Bromphenole. *Monatsh. Chem.*, 94 (1963) 227–242.

10. CARBOHYDRATES

BROCKMANN, H. AND WAEHNELDT, T.: Rhodinose, eine neue Tridesoxyhexose. *Naturwissenschaften*, 50 (1963) 43.

KUHN, R. AND WIEGANDT, H.: Die Konstitution der Ganglio-N-tetraose und des Gangliosids G_1. *Chem. Ber.*, 96 (1963) 866–880.

WOLFROM, M. L., HORTON, D. AND HUTSON, D. H.: Thiosugars. II. Rearrangement of 2-(3, 4, 6-tri-O-acetyl-2-amino-2-deoxy-(β-D-glucopyranosyl)-2-thiopseudourea. *J. Org. Chem.*, 28 (1963) 845–847.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

KAUFMANN, H. P. AND DAS, B.: Die Dünnschicht-Chromatographie auf dem Fettgebiet. IX. Die Analyse der Esterwachse. *Fette, Seifen, Anstrichmittel*, 65 (1963) 398–402.

12. ORGANIC PEROXIDES

SCHAUENSTEIN, E. AND ESTERBAUER, H.: Über die Reaktion von $\Delta^{9,12}$-Linolsäureester in Wasser. 6. Mitt. Chromatographische Untersuchungen über die Zusammensetzung des Präparates "LHPO" und die Isolierung der einzelnen tumorwirksamen Hydroperoxydkomponenten. *Monatsh. Chem.*, 94 (1963) 164–174.

13. STEROIDS

KORZUN, B. P. AND BRODY, S.: Thin-layer chromatographic identity test for steroids in sesame oil preparations. *J. Pharm. Sci.*, 52 (1963) 206–207.

MÜHLE, H. AND TAMM, CH.: Über cyclische β-Diketone. 4. Mitt. Synthese und Eigenschaften von 2,2-Dimethyl-, 4,4-Dimethyl-, und 2,2,4,4-Tetramethylcholestandion-(1,3). *Helv. Chim. Acta*, 46 (1963) 268–282.

SCHWARZ, V.: Methoden zur Trennung von Naturstoffen. IX. Chromatographie von Steroidverbindungen an einer Magnesiumsilicat-schicht ohne Bindemittel. *Pharmazie*, 18 (1963) 122–124 — R_F values of 51 steroid derivatives in 10 systems.

SCHWARZ, V. AND SYHORA, K.: Steroid-Derivate XXII. Chromatographie polarer Steroide an einer Aluminiumoxydschicht. *Collection Czech. Chem. Commun.*, 28 (1963) 101–106 — R_F values of 19 pregnane derivatives and 12 steroid acids.

WARNHOFF, E. W.: α-Halo-ketones. III. The epimeric 2,2-bromo-chlorocholestan-3-ones and the stereochemistry of ketone halogenation. *J. Org. Chem.*, 28 (1963) 887–894 — R_F values of 10 compounds.

14. STEROID GLYCOSIDES

JANIAK, P. ST., WEISS, EK., V. EUW, J. AND REICHSTEIN, T.: Die Konstitution von Adynerin. *Helv. Chim. Acta*, 46 (1963) 374–392.

18. AMINO ACIDS

BENTLEY, J. P. AND JACKSON, D. S.: *In vivo* incorporation of labeled amino acids during early stages of collagen biosynthesis. *Biochem. Biophys. Res. Commun.*, 10 (1963) 271–276.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

LEGLER, G. AND TSCHESCHE, R.: Die Isolierung von N-Methyltryptamin, 5-Methoxy-N-methyltryptamin und 5-Methoxy-N,N-dimethyltryptamin aus der Rinde von *Piptadenia peregrina* Benth. *Naturwiss.*, 50 (1963) 94–95.

24. ORGANIC SULPHUR COMPOUNDS

CAMPAIGNE, E. AND GEORGIADIS, M.: Thiocarbonyls. IX. The use of thin layer chromatography in the separation of the isomeric trithiofluorobenzaldehydes. *J. Org. Chem.*, 28 (1963) 1044–1048 — R_F values of 18 compounds.

KHO, T. B. AND KLEIN SHELDON: Thin-layer chromatography in drug analysis. II. Procedure for the identification of various N^4-substituted sulfonamides. *J. Pharm. Sci.*, 52 (1963) 404–406.

25. ORGANIC PHOSPHORUS COMPOUNDS

ROBINSON, N. AND PHILLIPS, B. M.: Quantitative thin-layer chromatography of serum phospholipids. *Clin. Chim. Acta*, 8 (1963) 385–392.

30. SYNTHETIC AND NATURAL DYES

ANWAR, M. H.: Separation of plant pigments by thin-layer chromatography. *J. Chem. Educ.*, 40 (1963) 29–31.

ROLLINS, C.: Thin-layer chromatographic separation of leaf pigments. *J. Chem. Educ.*, 40 (1963) 32

32. PHARMACEUTICAL APPLICATIONS

BRAUN, B. AND KÜMMEL, H. J.: Über die Verwendungsmöglichkeit von Kunststoffbehältern zur Aufbewahrung von Blut und Infusionslösungen. *Deut. Apotheker-Ztg.*, 103 (1963) 467–474.

GÄNSHIRT, H.: Nachweisverfahren für dünnschichtchromatographisch getrennte Wirkstoffe von Antineuralgie- und Schlaftabletten. *Arch. Pharm.*, 296 (1963) 73–79 — detection reactions for 16 compounds using 7 reagents.

GÄNSHIRT, H.: Untersuchung zur quantitativen Auswertung der Dünnschichtchromatographie. III. Bestimmung der in einem pharmazeutischen Kombinationspräparat enthaltenen Komponenten: Coffein, Mandelsäurebenzylester, Amidopyrin und Phenacetin. *Arch. Pharm.*, 296 (1963) 129–134 — quantitative analysis after elution.

HÖRHAMMER, L., WAGNER, H. AND KÖNIG, H.: Neue Methoden im pharmakognostischen Unterricht. 6. Mitt. Chromatographie der Inhaltsstoffe von Strophantussamen und ihrer Arzneizubereitungen. *Deut. Apotheker-Ztg.*, 103 (1963) 502–504 — R_F values of 11 components.

HÖRHAMMER, L., WAGNER, H. AND LAY, B.: Neue Methoden im pharmakologischen Unterricht. 5. Mitt. Zur Chromatographie von *Folia Farfarae* und *Folia Petasites*. *Deut. Apotheker-Ztg.*, 103 (1963) 429–431.

ŠARŠÚNOVÁ, M. AND SCHWARZ, V.: Dünnschicht-Chromatographie ohne Bindemittel in der Arzneimittelanalyse. *Pharmazie*, 18 (1963) 34–37.

ŠARŠÚNOVÁ, M. AND SCHWARZ, V.: Einige Möglichkeiten der Ausnutzung der Dünnschicht-Chromatographie ohne Bindemittel in der Arzneimittelanalyse. *Pharmazie*, 18 (1963) 207–209.

34. RADIOACTIVE COMPOUNDS

KARLSON, P., MAURER, R. AND WENZEL, M.: Eine Mikromethode zur Markierung von Steroiden und von Ecdyson mit Tritium. *Z. Naturforsch.* 18b (1963) 219–224 — pregnane derivatives.

Gas Chromatography

INTRODUCTION

One of the most urgent problems facing the scientist, whose interests are not exclusively concerned with a specialised subject, is to maintain a survey over the rapidly increasing number of papers on gas chromatography, which, due to the exploitation of gas chromatography in various branches of science and industry, are scattered throughout the literature and hence numerous papers, containing new results or methods cannot be identified as such from their title and thus escape attention.

Gas chromatography has attained as a quantitative and at the same time extremely precise chromatographic method, an independent position amongst other chromatographic methods and thus already two independent bibliographies are published, the *Gas Chromatography Abstracts*, (Butterworths, London) and the *Gas Chromatography Abstracting Service* (Preston Technical Abstracts Co., Evanston, Ill., U.S.A.), which are doing their best to give the documentation of gas chromatography full coverage.

This service is useful and needed. But we are of the opinion that with the extending exploitation of gas chromatography, its links with other types of chromatography will increase. The growing number of scientists working in this field would wish to be effectively and quickly informed of such publications. We consider it of importance to stimulate this trend with the selection of papers in a manner similar to the bibliography of paper chromatography, started successfully in the *Journal of Chromatography*. Thus we intend using a classification aligned on that of paper and thin-layer chromatography, only the first sections being specific to the requirements of gas chromatography.

The selection will comprise 50–60 % of the papers dealing with gas chromatography and will be compiled from 120 scientific journals and three abstracting journals (*Chemical Abstracts*, *Analytical Abstracts* and *Referativnyi Zhurnal, Khimiya*) and will deal with topics of importance on the theory, techniques and properties of the compounds chromatographed. The information given in some papers will be summarized in a few words.

JAROSLAV JANÁK

1. REVIEWS AND BOOKS

DE SAINT-RAT, L. AND BERTRAND, D.: First international symposium on the analysis of foods by gas chromatography. *J. Gas Chromatog.*, 1, No. 4 (1963) 31–33.

JANÁK, J.: Gas chromatography symposium, Brno, Czechoslovakia, July 1962. *J. Gas Chromatog.*, 1, No. 4 (1963) 6–10 — abstracts of 42 papers.

PRESTON, S. T., Jr. AND HYDER, G.: Gas chromatography patents, 1952–1963. Part I, United States Patents. *J. Gas Chromatog.*, 1, No. 3 (1963) 22–26.

PRESTON, S. T., Jr. AND HYDER, G.: Gas chromatography patents, 1952–1963. Part I (continued). United States Patents. *J. Gas Chromatog.*, 1, No. 4 (1963) 26–30.

WURST, M.: (Gas chromatography. Part 1. Methodics of qualitative analysis.) *Chem. Listy*, 57 (1963) 113–129 — a review with 86 references.

2. FUNDAMENTALS, THEORY AND GENERAL

2a. *Gas–liquid systems*

DEFORD, D. D., LOYD, R. J. AND AYERS, B. O.: Studies on the efficiencies of packed gas chromatographic columns. *Anal. Chem.*, 35 (1963) 426–429.

GIDDINGS, J. C.: Advances in the theory of plate height in gas chromatography. *Anal. Chem.*, 35 (1963) 439–449.

GIDDINGS, J. C.: Plate height of nonuniform chromatographic columns. Gas compression effects, coupled columns, and analogous systems. *Anal. Chem.*, 35 (1963) 353–356.

GIDDINGS, J. C.: Generalized nonequilibrium theory of plate height in large-scale gas chromatography. *J. Gas Chromatog.*, 1, No. 4 (1963) 38–42.

KNOX, J. H. AND MCLAREN, L.: The spreading of air peaks in capillary and packed gas chromatographic columns. *Anal. Chem.*, 35 (1963) 449–454.

MARTIN, R. L.: Adsorption of solutes at the liquid–gas interface as measured by gas chromatography and Gibbs equation. *Anal. Chem.*, 35 (1963) 116–117.

PERRETT, R. H. AND PURNELL, J. H.: Contribution of diffusion and mass transfer processes to efficiency of gas liquid chromatography columns. *Anal. Chem.*, 35 (1963) 430–439.

SCOTT, R. P. W.: Gas liquid chromatography. Temperature changes during passage of a solute through a theoretical plate. *Anal. Chem.*, 35 (1963) 481–488 — deformation of peaks through temperature changes during sorption.

ZHUKHOVITSKIĬ, A. A., TURKELTAUB, N. M., GAYER, M., LAGASHKINA, M. N., MALYASOVA, L. A. AND SHLEPUZHNIKOVA, G. P.: (Vakantochromatography). *Zavodsk. Lab.*, 29 (1963) 8–13 — new variant; injection of pure carrier gas in the mixture passing through the column.

2b. *Gas–solid systems*

SZEPESY, L. AND ILLÉS, V.: Adsorption of gases and gas mixtures. I. Measurement of the adsorption isotherms of gases on active carbon up to pressures of 1000 torr. *Acta Chim. Acad. Sci. Hung.*, 35 (1963) 36–51.

2c. *Thermodynamics and relations*

AYERS, B. O., LOYD, R. J. AND SANFORD, R. A.: Data systemization for the selection and optimization of chromatographic columns. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

BEREZKIN, V. G. AND SIDOROVA, N.V.: (Calculation of the relative retention times of compounds in composite chromatographic columns. *Neftekhimiya*, 3 (1963) 144–148 — mean errors 5–10% for hydrocarbons.

GENKIN, A. N., OGORODNIKOV, S. K., KOGAN, V. B., NEMCOV, M. S. AND PRESMAN, B. I.: (Effect of polar compounds on relative volatility of C_5 hydrocarbons). *Zh. Prikl. Khim.*, 36 (1963) 142–147 — agreement between GLC and static measurements.

HENLY, R. S., ROSE, A. AND SWEENY, R. F.: The partition coefficient as a function of concentration in gas–liquid chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

LANGER, S. H. AND PURNELL, J. H.: A gas–liquid chromatographic study of the thermodynamics of solution of some aromatic compounds. *J. Phys. Chem.*, 67 (1963) 263–270.

ZHUKHOVITSKIĬ, A. A. AND TURKELTAUB, N. M.: (On the chromatographic analysis of impurities). *Neftekhimiya*, 3 (1963) 135–143 — theory of trace analysis in the case of partially overlapping peaks.

2d. *General*

HORNING, E. C., MADDOCK, K. C., ANTHONY, K. V. AND VANDENHEUVEL, W. J. A.: Quantitative aspects of gas chromatographic separation in biological studies. *Anal. Chem.*, 35 (1963) 526–532 — methyl esters of long-chain fatty acids and steroids.

Mashiko, Y., Araki, S., Maruyama, M. and Takanishi, T.: GCDC cards coding system. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

Nelson, D. C. and Paull, D. L.: Absolute calibration of gas chromatographic apparatus from ion chamber measurements. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

3. TECHNIQUES I

3a. Detectors

Camin, D. L., King, R. W. and Shawhan, S. D.: Capillary column gas chromatography using micro-thermal conductivity cells as detectors. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

Dorsey, J. A., Hunt, R. H. and O'Neal, M. J.: Rapid-scanning mass spectrometry. Continuous analysis of fractions from capillary gas chromatography. *Anal. Chem.*, 35 (1963) 511–515 — research methods for identification of C_9 hydrocarbons.

Findeis, A. F. and Williams, F. W.: Gas analysis by Geiger pulse attenuation. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31–April 5, 1963.*

Folmer, Jr., O. F., Yang, K. and Perkins, Jr., G.: Use of catalytic combustion filaments for qualitative gas chromatography. *Anal. Chem.*, 35 (1963) 454–459 — signal of different types of compounds corresponding with filament current.

Gudzinowicz, B. J. and Smith, W. R.: New radioactive gas chromatographic detector for identification of strong oxidants. *Anal. Chem.*, 35 (1963) 465–467 — ^{85}Kr quinol clathrate sensing element for Br_2,NO_2,OF_2,Cl_2,NO_2F_2 and NO_2Cl.

Johns, T.: Application of an electrical conductivity detector in gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

Judson, C. M., McKinney, C. R. and Howard, R. F.: Use of a conventional mass spectrometer for gas chromatography monitoring. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

Karmen, A., McCaffrey, I., Winkelman, J. W. and Bowman R.L.: Measurement of tritium in the effluent of a gas chromatography column. *Anal. Chem.*, 35 (1963) 536–542 — combustion to ^3H_2O and reaction on Fe in H_2 produce ^{3}H-labelled H_2-stream; detection on anthracene crystals.

Lipsky, S. R., and Shahin, M. M.: Sensitive ionization system for the detection of permanent gases and inorganic vapours by gas chromatography. *Nature*, 197 (1963) 625–626 — cross-section microdetector; sensitivity 10^{-10} to 10^{-11} g/s for permanent gases, 10^{-7} to 10^{-9} g/s for rare gases and N_2.

Lovelock, J. E.: Electron absorption detectors and technique for use in quantitative and qualitative analysis by gas chromatography. *Anal. Chem.*, 35 (1963) 474–481 — anomalous behaviour of detectors is discussed.

Lovelock, J. E., Shoemake, G. R. and Zlatkis, A.: Sensitive ionization cross-section detector for gas chromatography. *Anal. Chem.*, 35 (1963) 460–465 — 100-fold increase of sensitivity.

Perkins, Jr., G., Laramy, R. E. and Lively, L. D.: Flame response in the quantitative determination of high molecular weight paraffins and alcohols by gas chromatography. *Anal. Chem.*, 35 (1963) 360–362.

Reynolds, R. G. and Monkman, J. L.: The calibration of flame ionization detectors. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

Schwartz, R. D., Brasseaux, D. J. and Shoemake, G. R.: Capillary column gas–liquid chromatography with thermal conductivity detectors. *J. Gas Chromatog.*, 1, No. 1 (1963) 32–33 — thermistors or hot wires; nylon capillaries.

Shahin, M. M. and Lipsky, S. R.: The mechanisms of operation of a new and highly sensitive ionization system for the detection of permanent gases and organic vapors by gas chromatography. *Anal. Chem.*, 35 (1963) 467–474 — 200 mC $^3H_2$; argon; potential 1–2 V at 150–200°; signals 10^{-11} A for approx. 0.1–1 μl of gases.

Sloane, H., Johns, T. and Ulrich, W.: Infrared examination of micro samples from gas chromatographic effluent. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963* —micro-reflectance attachment for I.R. examination.

Sternberg, J. C. and Bochinski, J.: Gaseous electronic detectors. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

3b. Column performance and filling studies

Ackman, R. G. and Burgher, R. D.: The effect of detector block fouling on observed separations in gas–liquid chromatography. *Anal. Chem.*, 35 (1963) 413–414.

Averill, W.: In-place coating of the solid support for gas chromatography. *J. Gas Chromatog.*, 1, No. 1 (1963) 35 — solution of stationary phase is forced directly through the filled column.

Bayer, E., Hupe, K. P. and Mack, H.: Filling of analytical and preparative columns for gas chromatography. *Anal. Chem.*, 35 (1963) 492–496 — columns 5–10 cm I.D.; samples of 500 g.

BOMBAUGH, K. J.: Improved efficiency in gas chromatography by molecular sieve flour. *Nature*, 197 (1963) 1102–1103 — sieve flour mixed with Chromosorb.

BRESLER, S. E., DOBYTHSHIN, D. P. AND POPOV, A. G.: (Use of wide porous glass as solid support for gas–liquid chromatography). *Zhr. Prikl. Khim.*, 36 (1963) 66–74 — pore diameters of 1000–3500 Å and surface of 14.5–23 m^2/g; better than firebrick.

CARTER, H. V.: Micro-packed columns for gas chromatography. *Nature*, 197 (1963) 684–685 — columns 0.25–0.5 mm I.D. packed with glass beads of 0.04 mm; thermistor; samples of 10^{-5} g.

CIEPLINSKI, E. W., KABOT, F. J. AND ETTRE, L. S.: Application of dual column gas chromatographic systems for the solution of complex problems. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

ETTRE, L. S.: Possibilities of investigating and expressing column efficiencies. *J. Gas Chromatog.*, 1, No. 2 (1963) 36–47.

ETTRE, L. S., CIEPLINSKI, E. W. AND AVERILL, W.: Application of open tubular (Golay) columns with larger diameter. *J. Gas Chromatog.*, 1, No. 2 (1963) 7–16.

FETT, E. R.: Backflush applied to capillary column–flame ionization detector gas chromatography systems. *Anal. Chem.*, 35 (1963) 419–420.

GUIOCHON, G.: Influence of injection time on the efficiency of gas chromatography columns. *Anal. Chem.*, 35 (1963) 399–400.

HALÁSZ, I. AND HORVÁTH, C.: Thin-layer graphited carbon black as the stationary phase for capillary columns in gas chromatography. *Nature*, 197 (1963) 71–72 — carbon black (73 m^2/g) in silvered copper capillaries; symmetrical peaks for C_6 hydrocarbons and oxygenated compounds at 175–245°.

HALÁSZ, I. AND HORVÁTH, C.: Open tube columns with impregnated thin-layer support for gas chromatography. *Anal. Chem.*, 35 (1963) 499–505 — inorganic supports (β-Fe_2O_3, silicates) with liquid phases.

JENTZSCH, D. AND HÖVERMANN, W.: New application of Golay columns with special wall treatments. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

LYSYJ, I. AND NEWTON, P. R.: Evaluation of gas chromatographic columns for the separation of fluorinated materials. *Anal. Chem.*, 35 (1963) 90–92 — retention data for SiF_4, CF_4, NF_3, HF, SF_6, F_2, ClF_3 and some freons.

MANKA, D. P.: Treatment of columns in gas chromatographic analysis of aqueous solutions. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

MCINTYRE, E. A., ROONEY, T. B., CURREN, W. J. AND AZNAVOURIAN, W.: Determining conditions for low temperature analysis of high boiling compounds. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

MORTIMER, J. V. AND GENT, P. L.: Use of modified Bentone-34 for the gas chromatographic separation of aromatic hydrocarbons. *Nature*, 197 (1963) 789–790 — silicone oil as modifier; separation of *m*- and *p*-xylene (α = 1.13 at 70°).

OTTENSTEIN, D. M.: Column support materials for use in gas chromatography. *J. Gas Chromatog.*, 1, No. 4 (1963) 11–23.

PETITJEAN, D. L. AND LEFTAULT, JR. C. J.,: Oxide-coated aluminium tubing for capillary gas chromatography. *J. Gas Chromatog.*, 1, No. 3 (1963) 18–21.

PORTER, R. S., HINKINS, R. L., TORNHEIM, L. AND JOHNSON, J. F.: Computer optimization of mixed liquid phases for gas chromatography. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31–April 5, 1963.*

PRESTON, S. T., JR.: Temperature limitations of stationary phases used in gas chromatography. *J. Gas Chromatog.*, 1, No. 3 (1963) 8–10.

REILLEY, C. N., HILDEBRAND, G. P., PAPA, L. J. AND NORTEMAN, Jr., W. E.: Gas chromatography with mixed bed packing. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31–April 5, 1963.*

ROGOZINSKI, M. AND WARSHAVSKY, A.: Comments on cyanoethylated polyols as gas chromatographic stationary phases. *J. Gas Chromatog.*, 1, No. 3 (1963) 30.

SCHWARTZ, R. D., BRASSEAUX, D. J. AND SHOEMAKE, G. R.: Sol-coated capillary adsorption columns for gas chromatography. *Anal. Chem.*, 35 (1963) 496–499 — deposition of silica (particle diameter approx. 20 mμ) from colloidal sol.

SIDEMAN, S.: Separation with infinite circular columns in gas chromatography. *Chem. Eng. Sci.*, 18 (1963) 95–98.

STERNBERG, J. C. AND POULSON, R. E.: Principles of low pressure drop packed columns. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

SUPINA, W. R.: The use of organosilicon polyesters as stationary phases for gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

THOMA, J. A.: Application and theory of unidimensional multiple chromatography. *Anal. Chem.*, 35 (1963) 214–224.

VÁRADI, P. F. AND ETTRE, K.: Vacuum outlet gas chromatography. *Anal. Chem.*, 35 (1963) 410–412.

3c. Apparatus, accessories and materials for GC

ANDREW, T. D., PHILLIPS, C. S. G. AND SEMLYEN, J. A.: Some applications of an R. F. oscillator in gas chromatography. *J. Gas Chromatog.*, 1, No. 1 (1963) 27–30 — details of construction, application as flash vaporizer, as pyrolysis assembly, as argon detector modifier.

JAMES, A. T. AND PIPER, E. A.: A compact radiochemical gas chromatograph. *Anal. Chem.*, 35 (1963) 515–520 — labelled fatty acids detected as $^{14}CO_2$ and $^{3}H_2$ after combustion.

HACHENBERG, H., JUNGHANNS, M. AND REININGER, H.: (Automatic range switching device for gas chromatography). *Brennstoff-Chem.*, 44 (1963) 6–8 — full description of electric circuit.

HARALDSON, L. AND THORNEMAN, T.: Sampling valve for small volumes of gas. *Mikrochim. Acta*, No. 1 (1963) 14–18 — for 1–120 μl samples.

JENKINS, P.: A reliable system for the introduction of samples to gas chromatographic columns, particularly suited for quantitative capillary column analysis. *Nature*, 197 (1963) 72–73.

KERTZMAN, J.: The Gilbarco gas purifier as applied to analytical instrumentation. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

KIRKLAND, J. J.: A critical study of fluorocarbons as solid supports in gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

MCCOMNAS, D. B. AND GOLDFIEN, A.: A device for the introduction of sub-microgram quantities of solids into a gas chromatograph. *Anal. Chem.*, 35 (1963) 263–264.

MERRITT, J., COMENDANT, F., ABRAMS, S. T. AND SMITH, V. N.: Process gas chromatograph with ultraviolet detector. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

NĚMEC, L. AND PETZELT, B.: (Universal gas chromatograph II). *Chem. Listy*, 57 (1963) 364–371 — combined packed and capillary columns with flame ionization detector.

NOVÁK, J.: High temperature gas chromatograph. *Tech. Digest*, 5, No. 2 (1963) 38–40.

NOVÁK, J. AND JANÁK, J.: (Working properties of prototype of high temperature gas chromatograph Chrom. II). *Chem. Listy*, 57 (1963) 371–389.

RYABCHIKOVA, G. G., SIBIRSKAYA, G. K., GLAZUNOV, P. YA. AND GRACHEV, A. I.: (Semi-automatic sampling device for gas chromatography). *Zavodsk. Lab.*, 29 (1963) 243–244 — for volumes 1–5, 0.3–1 and 0.01–0.3 ml; designs.

RYABCHIKOVA, G. G., SIBIRSKAYA, G. K., GLAZUNOV, P. YA. AND GRACHEV, A. I.: (Sampling device for gaseous samples in gas chromatographic analysis). *Zavodsk. Lab.*, 29 (1963) 244 — sampling from molten glass capillaries.

SLAVÍČEK, I. AND ŘÍHA, F.: (Analog integrator for use in chemical laboratories and plants). *Chem. Prumysl*, 13 (1963) 24–26 — AC speed servomechanism with depressed ground frequency.

SPOLNICKI, J. AND CROOKS, W. M.: A chromatography unit, with automatic sampling, for kinetic studies. *J. Appl. Chem. (London)*, 13 (1963) 12–16 — details of sampling device and amplifier for flame ionization detection.

SZYMANSKI, H. A.: Gas chromatography using induction heated columns. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

WILKINSON, J. AND HALL, D.: A device for transferring small volumes of gas from a vacuum line to a gas chromatograph. *J. Chromatog.*, 10 (1963) 239–242 — for 0.04–1.0 ml samples; accuracy $\pm$ 5% for 0.1 ml sample.

4. TECHNIQUES II

4a. Preparative-scale GC

DIMICK, K. P. AND TAFT, E. M.: A multiple process used in automatic preparative gas chromatography. *J. Gas Chromatog.*, 1, No. 3 (1963) 7.

GIDDINGS, J. C.: Principles of column performance in large scale gas chromatography. *J. Gas Chromatog.*, 1, No. 1 (1963) 12–21 — resolution loss is caused by the consistent increase in zone velocity in second part of column.

GOLAY, M. J. E., HILL, H. I. AND NOREM, S. D.: Recirculated column for preparative scale gas chromatography. *Anal. Chem.*, 35 (1963) 488–491 — arrangement for elimination of all faster and slower components.

SPENCER, S. F. AND KAUSS, J. M.: Applications of automatic preparative gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

TAFT, E. M. AND DIMICK, K. P.: An automatic preparative gas chromatograph. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

WIEGLEB, H. AND PRINZLER, H.: (Automation of preparative gas chromatographic unit). *Chem. Tech. (Berlin)*, 15 (1963) 98–101 — detailed electronic scheme.

4b. Programmed temperature GC

MARCALI, K.: Trace analysis by temperature-gradient gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

McEWEN, D. J.: Temperature programmed columns in gas chromatography. Comparison of packe dand capillary columns for the analysis of dilute hydrocarbon gas mixtures. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31–April 5, 1963.*

MERRITT, C., Jr. AND WALSH, J. T.: Programmed cryogenic temperature gas chromatography applied to the separation of complex mixtures. *Anal. Chem.*, 35 (1963) 110–113 — temperature —196° to ambient; volatile components from irradiated ground beef and roasted coffee beans.

OHLINE, R. W. AND DEFORD, D. D.: Chromathermography, the application of moving thermal gradients to gas liquid partition chromatography. *Anal. Chem.*, 35 (1963) 227–234.

4d. Special microtechniques

AMY, J. W. AND DIMICK, K. P.: Multichannel gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

BOCHINSKI, J. H., JOHNS, T. AND PORTER, J. A.: Pre-extractive techniques as applied to gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

DE MOURGUES, L.: (Applications of gas chromatography to the study of solid catalysts). *Chim. Anal. (Paris)*, 45 (1963) 103–110 — microcatalytic device; measuring of adsorption isotherm.

DVORETZKY, I., RICHARDSON, D. B. AND DURRETT, L. R.: Applications of the methylene insertion reaction to component analysis of hydrocarbons. *Anal. Chem.*, 35 (1963) 545–549 — CH_2 from photolysis of diazomethane.

ETTRE, K. AND VÁRADI, P. F.: Pyrolysis-gas chromatographic technique. Effect of temperature on thermal degradation of polymers. *Anal. Chem.*, 35 (1963) 69–73 — new pyrolysis device is described.

MERRITT, C., JR.: Qualitative gas chromatographic analysis. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31–April 5, 1963.*

PARSONS, M. L., PENNINGTON, S. N. AND WALKER, J. M.: A rapid method for nitrogen analysis in organic compounds and associated materials by gas chromatography. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31–April 5, 1963.*

ZHUKHOVITSKIĬ, A. A., TURKELTAUB, N. M., KANTSEEVA, O. A., NAUMOVA, V. V. AND RYABCHUK, L. N.: (Stepwise chromatography). *Zavodsk. Lab.*, 29 (1963) 14–18.

4e. Automation

VALSTAR, J. E. AND PERCY, L. E.: Application of intermittent process analyzers to closed loop control. *J. Gas Chromatog.*, 1, No. 2 (1963) 24–27.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

FUTRELL, J. H. AND TIERNAN, T. O.: Inhibition of the argon-sensitized radiolysis of propane by hydrogen. *J. Chem. Phys.*, 38, No. 1 (1963) 150–154.

INNES, W. B., BAMBRICK, W. E. AND ANDREATCH, A. K.: Hydrocarbon gas analysis using differential chemical absorption and flame ionization detectors. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

JARZYŇNSKA, M. AND MIRECKA, T.: (Separation of unsaturated hydrocarbons C_2–C_4 by means of gas–liquid chromatography on Janák's apparatus). *Chem. Anal. (Warsaw)*, 8 (1963) 91–94 — $AgNO_3$ in polyethylene glycol.

MIQUEL, R. AND BÉNARD, C.: (Chromatographic separation of hydrogen and gaseous hydrocarbons at normal temperatures). *Compt. Rend.*, 256 (1963) 940–941.

ROBERTS, L. R.: Analysis of multicomponent hydrocarbon systems containing large amounts of nitrogen. *J. Gas Chromatog.*, 1, No. 3 (1963) 14–17.

5b. Other hydrocarbons

ARCHER, E. D., SHIVELY, J. H. AND FRANCIS, S. A.: Analysis of C_7 olefins by a combination of gas chromatography and nuclear magnetic resonance spectroscopy. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

DORING, C. E. AND HAUTHAL, H. G.: (Gas chromatographic investigation of C_6 hydrocarbons). *J. Prakt. Chem.*, 19 (1963) 17–32.

FERRAND, R.: (Possibilities of applications of gas chromatography to the study of coal tar oils). *Chim. Anal. (Paris)*, 45 (1963) 133–134 — 320°, tetracene, perylene, picene, coronene.

FRANCIS, S. A. AND ARCHER, E. D.: Identification of triisobutylene and tetraisobutylene by a combination of gas chromatography and nuclear magnetic resonance techniques. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

HILDEBRAND, G., PEPER, C. AND DAHLKE, B.: (Separating liquids for the analysis of solid paraffin hydrocarbons by means of high-temperature gas chromatography). *Chem. Tech.* (*Berlin*), (1963) 147–150.

KNIPSCHILD, J. AND PAGNIER, G.: (Direct chromatographic determination of naphthalene and methylnaphthalene in gas.) *Brennstoff-Chem.*, 44 (1963) 8–10 — in mg/m^3 range.

SPEARS, A. W., LASSITER, C. W. AND BELL, J. H.: Quantitative determination of alkanes in cigarette smoke. *J. Gas Chromatog.*, 1, No. 4 (1963) 34–37.

SPENCER, S. F.: Rapid separation of xylenes and ethylbenzene by gas chromatography using packed columns. *Anal. Chem.*, 35 (1963) 592–594 — 5% Bentone-34 + 5% diisodecylphthalate on Chromosorb W at 75°.

VAISBERG, K. M., KRUGLOV, E. A., KHABIBULLIN, M. F. AND SHABALIN, I. I.: (Study of composition of different types of naphthalene by means gas–liquid chromatography). *Koks i Smola*, 3 (1963) 44–47.

5c. Halogen derivatives of hydrocarbons

BECKMAN, H. AND BEVENUE, A.: The effect of the column tubing composition on the recovery of chlorinated hydrocarbons by gas chromatography. *J. Chromatog.*, 10 (1963) 231–233 — all-glass or all-quartz apparatus is preferred; recoveries: quartz > aluminium > stainless steel > copper.

JUBY, P. F. AND MARION, L.: The origin of carbon atoms 2, 3, and 7 of ricinine. *Can. J. Chem.*, 41 (1963) 117–122.

MASHIKO, Y., KANBAYASHI, U., NUKADA, K., SUZUKI, T., TAKEDA, I. AND TOMITA, H.: Analysis of a mixture of chloropropanes and chloropropenes using a preparative scale gas chromatograph and a high resolution NMR spectrometer. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy March 4–8, 1963.*

6. ALCOHOLS

FRIEDMAN, R. L. AND RAAB, W. J.: Determination of tabacco humectants by gas–liquid chromatography. *Anal. Chem.*, 35 (1963) 67–69 — programmed temperature GC (50–200°) for polyols.

KOMERS, R. AND KOCHLOEFL, K.: Studie über gaschromatographische Trennung stereoisomerer Alkylcyclohexanole. *Collection Czech. Chem. Commun.*, 28 (1963) 46–54 — retention data of 38 compounds.

MLEJNEK, O.: (Identification of polyols in polyesters by means of gas–liquid chromatography). *Chem. Prumysl*, 13 (1963) 105–107 — as acetates at 100–140° in polyesters as well as in alkyd resins.

MLEJNEK, O. AND ADAMEC, V.: (Determination of small quantities of methyl alcohol in ethyl alcohol by means of gas–liquid chromatography). *Chem. Zvesti*, 17 (1963) 118–125 — all-glass flame ionization detector for concentrations of 10 p.p.m.; ethyl formate as internal standard.

PUSCHMANN, H.: (Application of gas chromatography in the examination of polyethylene glycols). *Fette, Seifen, Anstrichmittel*, 65 (1963) 1–5.

ROGOZINSKI, M., SHORR, L. M. AND WARSHAWSKY, A.: Gas chromatographic analysis of aqueous alcohols. II. Quantitative analysis of aqueous butanol solutions containing non-volatile salts. *J. Chromatog.*, 10 (1963) 114–116 — detailed injection block designed.

7. PHENOLS

KRUMBHOLZ, K. AND MEISSNER, W.: (Contribution to analysis of phenols in brown coal tar products). *Chem. Tech.* (*Berlin*), 15 (1963) 101–104 — simple phenols on silicone oil at 150°.

SPEARS, A. W.: Quantitative determination of phenols in cigarette smoke. *Anal. Chem.*, 35 (1963) 320–322.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

POLYAKOVA, T. A., SOKOLOVA, T. A. AND CARFIN, YA. A.: (Chromatographic determination of furan and carbon dioxide in products of decarboxylation of furfural). *Zavodsk. Lab.*, 29 (1963) 18–19 — tricresyl phosphate, 34°.

9. OXO COMPOUNDS

BAKER, J. M. AND KING, B. D.: Rapid blood ether determinations using gas chromatography. *Anesthesiology*, 24, No. 1 (1963) 122–122.

10. CARBOHYDRATES

ASPINALL, G. O.: Gas–liquid partition chromatography of methylated and partially methylated methyl glycosides. *J. Chem. Soc.*, (1963) 1676–1680.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

AVERILL, W.: Gas chromatography of free fatty acids using Golay columns. *J. Gas Chromatog.*, 1, No. 1 (1963) 22–22 — 10% trimer acid + 0.4% dinonylnaphthalenedisulphonic acid; retention data.

BROCKERHOFF, H., ACKMAN, R. G. AND HOYLE, R. J.: Specific distribution of fatty acids in marine lipids. *Arch. Biochem. Biophys.*, 100, No. 1 (1963) 9–12.

COLODZIN, M., BACHUR, N. R., WEISSBACH, H. AND UDENFRIEND, S.: Enzymatic formation of fatty acid amides of ethanolamine by rat liver microsomes. *Biochem. Biophys. Res. Commun.*, 10, No. 2 (1963) 165–170.

CRAIG, B. M. AND YOUNGS, C. G.: Fatty acid composition and glyceride structure in rats fed rapeseed oil or corn oil. *Can. J. Biochem. Physiol.*, 41, No. 1 (1963) 43–49.

GEHRKE, C. W. AND GOERLITZ, D. F.: Quantitative preparation of methyl esters of fatty acids for gas chromatography. *Anal. Chem.* 35 (1963) 76–80 — esterification by iodomethane with silver salts of acids in biological samples is advocated.

METCALFE, L. D.: The gas chromatography of fatty acids and related long-chain compounds on phosphoric acid treated columns. *J. Gas Chromatog.*, 1, No. 1 (1963) 7–11 — C_{10}–C_{18} fatty acids; nitriles, amides, sulfonated compounds mentioned.

PRIVETT, O. S., NADENICEK, J. D., WEBER, R. P. AND PUSCH, F. J.: Petroselinic acid and non-saponifiable constituents of parsley seed oil. *J. Am. Oil Chemist's Soc.*, 40 (1963) 28–30.

REYNOLDS, L. M. AND SMYTH, R. B.: The analysis of carboxylic acid congeners in distilled alcoholic beverages by gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

SHULGIN, A. T.: Composition of the myristicin fraction from oil of nutmeg. *Nature*, 197 (1963) 379–379.

STEIN, A., OPALKA, E. AND PECK, F.: Fatty acid analysis of brain tumors by gas chromatography. *Arch. Neurol.*, 8 (1963) 50–55 — C_{12}–C_{18} saturated and unsaturated fatty acids; 170°; argon.

ZEMAN, I. AND POKORNÝ, J.: Gas chromatographic analysis of cyclopentenyl fatty acids. *J. Chromatog.*, 10 (1963) 15–20 — retention volumes of C_{10}–C_{18} acids.

12. ORGANIC PEROXIDES

HYDEN, S.: Applications of pyrolysis-gas chromatography decomposition of dialkyl peroxides. *Anal. Chem.*, 35 (1963) 113–114 — errors of quantitative results are better than 10% of theory.

13. STEROIDS

CHAMBERLAIN, J. AND THOMAS, G. H.: Characterisation of steroid ketones by gas chromatography. *Biochem. J.*, 86 (1963) 3P.

HARTMAN, I. S. AND WOTIZ, H. H.: A method for the simultaneous separation of $C_{19}O_2$ and $C_{19}O_3$ 17-ketosteroids and progesterone metabolites by gas chromatography. *Steroids*, 1, No. 1 (1963) 33–38.

KNIGHTS, B. A. AND THOMAS, G. H.: ΔR_{Mg} values in the gas chromatography of steroids. *Chem. Ind. (London)* (1963) 43–44 — data for 34 steroids on QF-10065 at 250°.

KROMAN, H. S. AND BENDER, S. R.: An improved method for the gas chromatographic separation of estrogens. *J. Chromatog.*, 10 (1963) 111–112 — working limits of thermal stability.

LANDOWNE, R. A. AND LIPSKY, S. R.: The electron capture spectrometry of haloacetates: A means of detecting ultramicro-quantities of sterols by gas chromatography. *Anal. Chem.*, 35 (1963) 532–535 — responses for cholesteryl haloacetates.

NELSON, J. P.: Gas chromatography of selected pregnenes and pregnanes. *J. Gas Chromatog.*, 1, No. 3 (1963) 27–29.

TURNER, D. A., JONES, G. E. S., SARLOS, I. J., BARNES, A. C. AND COHEN, R.: Determination of urinary pregnanediol by gas chromatography. *Anal. Biochem.*, 5 (1963) 99–106 — 205°; silicone gum rubber + 50% cyanoethylpolysiloxane; argon; 14 retention data.

15. TERPENE DERIVATIVES

RUDLOFF VON, E.: Gas–liquid chromatography of terpenes. Part VII. The dehydration of P-menthan-8-ol. *Can. J. Chem.*, 41 (1963) 1–8.

SHULGIN, A. T.: Composition of the myristicin fraction from oil of nutmeg. *Nature*, 197 (1963) 379 — Silicon 710, 220°, isomerization of *cis*-isomyristin to the *trans*-isomer by GC.

16. NITRO AND NITROSO COMPOUNDS

DARLEY, E. F., KETTNER, K. A. AND STEPHENS, E. R.: Analysis of peroxyacyl nitrates by gas chromatography with electron capture detection. *Anal. Chem.*, 35 (1963) 589–591 — air pollution analysis; sensitivity ~ 10–50 p.p.b.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

FELTKAMP, H. AND THOMAS, K. D.: Die Trennung stereoisomerer Verbindungen durch Verteilung. IV. Mitt. Die gaschromatographische Trennung stereoisomerer cyclohexylamine. *J. Chromatog.*, 10 (1963) 9–14 — retention volumes of *cis-trans* isomers.
GRASSELLI, J. G., ROSS, B. L., HUBER, H. F. AND AUGL, J. M.: The gas chromatographic separation and infrared spectra of *cis-* and *trans*-1-cyanobuta-1,3-diene. *Chem. Ind.* (*London*), (1963) 162–163.
HISTA, C. AND BOMSTEIN, J.: Determination of benzyl chloroformate by gas chromatography of its amide. Extension of the principle to other acid chlorides. *Anal. Chem.*, 35 (1963) 65–67 — 0.25% Carbowax 1500 on glass beads, temperature programme 75–160°.
KUWADA, D. M.: Determination of water in hydrazine by gas chromatography. *J. Gas Chromatog.*, 1, No. 3 (1963) 11–13.
METCALFE, L. D.: The direct gas chromatographic analysis of long-chain quaternary ammonium compounds. *J. Am. Oil Chemist's Soc.*, 40 (1963) 25–27.
SZE, Y. L. AND BORKE, M. L.: Separation of lower aliphatic amines by gas chromatography. *Anal. Chem.*, 35 (1963) 240–242.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

CIEPLINSKI, E. W.: Prevention of peak tailing in the direct gas chromatographic analysis of barbiturates. *Anal. Chem.*, 35 (1963) 256–257.
PARKER, K. D., FONTAN, C. R. AND KIRK, P. L.: Improved gas chromatographic column for barbiturates. *Anal. Chem.*, 35 (1963) 418–419.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

BRUENNER, R. S., HAERTLE, W. R., LUNDQUIST, R. T., WNUK, R. J. AND RUBY, A.: The gas chromatographic separation and infrared identification of the 2,4- and 2,5-isomers of dimethyloxazoline. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

24. ORGANIC SULPHUR COMPOUNDS

PRINZLER, H.: (Contribution to the composition of petroleum and related materials). *Ropa Uhlie* 5 (13) (1963) 42–45 — retention data of some mercaptans, sulfides and thiophenes on dioctyl phthalate.
SPOREK, K. F. AND DANYI, M. D.: Detection and identification of mercaptans by gas–liquid chromatography after conversion to disulfides. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

26. METALLO-ORGANIC COMPOUNDS

BARRALL, E. M. AND BALLINGER, P.: Gas chromatographic analysis of lead alkyls with electron affinity detectors. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31–April 5, 1963.*
DAWSON, H. J., JR.: Determination of methyl-ethyl lead alkyls in gasoline by gas chromatography with an electron capture detector. *Anal. Chem.*, 35 (1963) 542–545 — 15% SE-30 silicone rubber on Chromosorb W + 8% NaOH; 160°; errors ± 0.01–0.08 g Pb/gallon.
PETERSON, J. I., KINDLEY, L. M. AND PODALL, H. E.: The synthesis and purification of organogermanium compounds for semiconductor application. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
PHILLIPS, J. R. AND STONE, F. G. A.: Purification of fluorocarbon organo-metallic and other sensitive compounds by vapour-phase chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
PRÖSCH, U. AND ZÖPEL, H. J.: (Gas-chromatographic analysis of methylethyl tin compounds.) *Z. Chem.* (*Leipzig*), 1 (1963) 97–100.

29. INSECTICIDES AND OTHER PESTICIDES

BECKMAN, H. F. AND BERKENKOTTER, P.: Gas chromatography of the reduction products of chlorinated organic pesticides. *Anal. Chem.*, 35 (1963) 242–246.
PETITJEAN, D. L. AND LANTZ, C. D.: Electron attachment determination of thiophosphate pesticides in the picogram range. *J. Gas Chromatog.*, 1, No. 2 (1963) 23.

31. PLASTICS AND THEIR INTERMEDIATES

LUCE, C. C., HUMPHREY, E. F., NORRISH, H. H. AND GUILD, L. V.: Analysis of polyester resins by gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

MASHIKO, Y. AND ECHIZEN, A.: Gas chromatographic rapid analysis of polymers. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
NEUMANN, E. W. AND NADEAU, H. G.: Qualitative identification of polyether and polyolefin type compounds by pyrolysis and gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
O'NEILL, H. J., PUTSCHER, R. E., DYNAKO, A. AND BOQUIST, C.: Pyrolysis studies of furfuryl alcohol resins by gas chromatography. *J. Gas Chromatog.*, 1, No. 2 (1963) 28–35.
PERCIVAL, D. F.: Analysis of polyester resins by gas chromatography. *Anal. Chem.*, 35 (1963) 236–238.
SHAPIRO, H., WEXLER, A. AND BRAKO, F.: Determination of residual monomers in polymer lattices by gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
TWEET, O. AND MILLER, W. K.: Determination of residual monomer in polymer emulsions by rapid distillation and gas chromatography. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31 – April 5, 1963.*

32. PHARMACEUTICAL APPLICATIONS

FONTAN, C. R., SMITH, W. C. AND KIRK, P. L.: Gas chromatography of the antihistamines. *Anal. Chem.*, 35 (1963) 591–591 — retention data of 14 antihistamines; 2 % wt. Carbowax 20M on acid-washed Chromosorb W + 20 % KOH; 190°.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

ARNETT, E. M. AND DUGGLEBY, P. M.: A practical method of deuterium analysis by gas chromatography – a progress report. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
ATTRILL, J. E., BOYD, C. M. AND MEYER, A. S., JR.: Gas chromatographic analysis of helium at reduced pressures. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31 – April 5, 1963.*
AZNAVOURIAN, W. AND MCINTYRE, E. A.: Gas chromatographic analysis of ammonia-fixed gas streams. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
BURNS, J. H.: Separation and detection of hydrogen, tritium hydride, and tritium at low-level tritium activities by gas chromatography. *J. Phys. Chem.*, 67 (1963) 535–536.
FARHI, L. E., EDWARDS, A. W. T. AND HOMMA, T.: Determination of dissolved N_2 in blood by gas chromatography and (a-A) N_2 difference. *J. Appl. Physiol.*, 18 (1963) 97–106 — Van Slyke gases through Dierite precolumn + Ascarite (CO_2 and H_2O) to the molecular sieve 2A column.
KARPATHY, O. C. I.: Carbon determination of ferrous alloys with a newly developed gas chromatograph. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
MANKA, D. P.: Complete gas chromatographic analysis of fixed gases with one detector and argon as carrier gas. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
MACKENZIE, R. G., REYNOLDS, R. G. AND MONKMAN, J. L.: The analysis of compressed air. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
RICHARDSON, R. D.: Process gas chromatography for the measurement of impurities in hydrogen as low as 0.5 p.p.m. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
VENUGOPALAN, M. AND KUTSCHKE, K. O.: Gas chromatographic separation of hydrogen isotopes on activated alumina. *Can. J. Chem.*, 41 (1963) 548–549 — carrier gas He; — 196°.

33b. Volatile inorganic substances

ISBELL, R. E.: Determination of hydrogen cyanide and cyanogen by gas chromatography. *Anal Chem.*, 35 (1963) 255–256.
JUVET, R. S., JR. AND TIVIN, F.: Analysis, solution thermodynamics, and purification for semiconductor purposes of inorganic compounds via gas chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
MOSHIER, R. W., SCHWARBERG, J. E., MORRIS, M. AND SIEVERS, R. E.: The quantitative aspects of the gas chromatography of certain metal coordinate compounds. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*
MYERS, H. W.: Application of gas chromatography to the study of chloroborane intermediates in the synthesis of diborane. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8 1963.*
PHILLIPS, C. S. G. AND TIMMS, P. L.: Some applications of gas chromatography in inorganic chemistry. *Anal. Chem.*, 35 (1963) 505–510 — Si and Si/Ge hydride, borazoles.

34. RADIOACTIVE COMPOUNDS

PASCAUD, M.: Contaminations en chromatographie gaz – liquide d'esters d'acides gras radioactifs sur phase stationnaire polyester. *J. Chromatog.*, 10 (1963) 125–130 — trans-esterification on polyethylene glycol adipate at 180° was proved.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

BARRALL, II, E. M., PORTER, R. S. AND JOHNSON, J. F.: Gas chromatographic analysis of poly-(ethylene ethyl acrylate) and poly (ethylene vinyl acetate) pyrolyzates. *Anal. Chem.*, 35 (1963) 73–76 — copolymers were analysed with absolute accuracy of 2%.

FEINLAND, R. AND CINCOTTA, J. J.: Determination of organic compounds in dilute aqueous solution by gas–liquid chromatography. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

GIRLING, G. W.: Evolution of volatile hydrocarbons from coal. *J. Appl. Chem. (London)*, 13 (1963) 77–91 — C_4– to C_{10} products of coal pyrolysis.

GUDZINOWICZ, B. J., CAMPBELL, R. H., DRISCOLL, J. L. AND MARTIN, H. F.: Gas chromatographic analysis of organic carbonates, aryl phosphines and posphine oxides and organo- and organo-bromoarsenic compounds. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

HINSVARK, O. N. AND THEALL, G.: Hydrogen reduction and determination of metal salts and oxides. Gas chromatographic measurement of reaction products. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

LIJINSKY, W., DOMSKY, I. I. AND MASON, G.: Analysis of tars and pyrolysates by gas chromatography programmed at moderate temperatures. *Natl. Am. Chem. Soc. Meeting, Los Angeles, Calif., March 31– April 5, 1963.*

MYERS, L. J.: Gas chromatographic analysis of liquids containing nonvolatile viscous materials. *Anal. Chem.*, 35 (1963) 119–120 — details of sample introduction cell.

NEUBAUER, N. R., SKRECKOSKI, G., WHITE, R. G. AND KANE, A. J.: Gas chromatographic analysis of free toluene diisocyanate in adducts with trimethylolpropane. *14th Pittsburgh Conf. Anal. Chem. Appl. Spectroscopy, March 4–8, 1963.*

PARKER, K. D., FONTAN, R. AND KIRK, P. L.: Rapid gas chromatographic method for screening of toxicological extracts for alkaloids, barbiturates, sympathomimetic amines and tranquilizers. *Anal. Chem.*, 35 (1963) 356–359.

QUIRAM, E. R.: Application of wide-diameter open tubular columns in gas chromatography. *Anal. Chem.*, 35 (1963) 593–595 — C_6–C_8 aromatic hydrocarbons, C_1–C_{16} alcohols in 150–250 m capillaries of 0.065 in. I.D. at 60–175°.

J. Chromatog., 12 (1963) 124–144

ERRATUM

D. G. ROUX, *J. Chromatog.*, 10 (1963) 473–478.

The stereochemistry of Compounds III and IV at C-4, has recently undergone revision. These now have the 2,4-*cis* and 2,3-*trans*-3,4-*trans* arrangements of substituents respectively [*cf.* S. E. DREWES AND D. G. ROUX, *Chem. Ind. (London)*, (1963) 532; C. P. LILLYA, S. E. DREWES AND D. G. ROUX, *Chem. Ind. (London)*, (1963) 783].

THEORETICAL PLATE HEIGHT IN HELICAL CHROMATOGRAPHIC COLUMNS

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Long columns for use in gas chromatography are usually coiled into a helix in order to conserve space, and there is some interest as to the extent such coiling should reduce the efficiency of the column. An earlier paper[1] attempted to analyze the situation and reached the conclusion that efficiency loss, or increase in plate height, would become serious with large tightly-coiled columns. This conclusion is certainly correct, but several questionable steps have been noted in the mathematical treatment which greatly reduce the significance of the analytical results obtained there.

In a coiled column, streamlines along the inside of the coil are shorter than those along the outside. The length distribution may be written in terms of the length l_0 along the centerline:

$$l = l_0 \left(\frac{R_0 + r \sin \theta}{R_0}\right) \tag{1}$$

Nomenclature is shown in Fig. 1. Since the pressure drop along all streamlines is the same for a given change in angle ϕ (*i.e.* no flow in r or θ directions exists) the axial carrier gas velocity may be written in terms of the center line velocity v_0:

$$v = v_0 \left(\frac{R_0}{R_0 + r \sin \theta}\right) \tag{2}$$

In ref. 1 this velocity gradient was properly written, but then cylindrical coordinates, applicable only to straight columns, were used in the subsequent mathematical

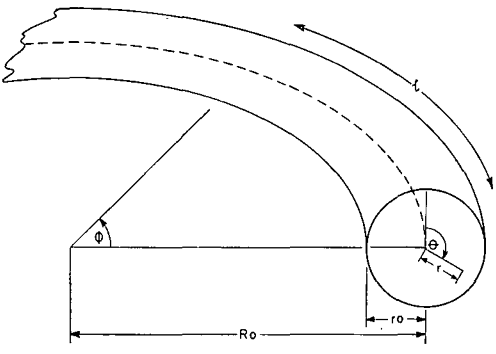

Fig. 1. Coordinate system.

treatment. This in effect said that a velocity gradient would exist but that all the streamlines were of equal length. A more realistic approach would be to use toroidal coordinates*. In this case the rate of accumulation of solute would be written as

$$\frac{\partial c}{\partial t} = \frac{RD}{(r)\,(R_0 + r \sin \theta)} \left[\frac{\partial}{\partial r} (r)\,(R_0 + r \sin \theta) \frac{\partial c}{\partial r} + \frac{\partial}{\partial \theta} \frac{(R_0 + r \sin \theta)}{r} \frac{\partial c}{\partial \theta} + \frac{r}{R_0 + r \sin \theta} \frac{\partial^2 c}{\partial \phi^2}\right] - \frac{v_0 R R_0}{(R_0 + r \sin \theta)^2} \frac{\partial c}{\partial \phi} \tag{3}$$

where c is overall concentration, t is time, R is fraction of component in mobile phase, and D is diffusivity (assumed constant here).

Assuming for the moment that the model actually used in ref. 1 (*i.e.* a straight column with the velocity distribution of a coiled column) is applicable to some physical system, two subsequent mathematical manipulations are questionable and lead to rather tenuous results. The first of these is the occasional elimination of the small term ε, defined by $c = c^* (1 + \varepsilon)$, where c^* is the concentration assuming lateral equilibrium. Although ε is small, so are the lateral diffusion effects which are sought, and any cancellation of small terms is more safely done in the final equation rather than in intermediate equations. The second is the use of incorrect averages in writing an overall material balance:

$$\frac{\partial c^*}{\partial t} = -\, v_0 R \frac{\partial c^*}{\partial z} + DR \frac{\partial^2 c^*}{\partial z^2} \tag{4}$$

The correct equation would be

$$\frac{\partial c^*}{\partial t} = -\frac{R}{\pi r_0^2} \iint v \frac{\partial c}{\partial z} r\mathrm{d}r\mathrm{d}\theta + \frac{DR}{\pi r_0^2} \iint \frac{\partial^2 c}{\partial z^2} r\mathrm{d}r\mathrm{d}\theta \tag{5}$$

Equation (4) may not be substituted for eqn. (5) with desired accuracy except in trivial cases.

By taking the two steps above, some rather untenable results were obtained. For instance the rate of accumulation s of solute due to lateral diffusion was said to be

$$s = (v - v_0)\, R \frac{\partial c^*}{\partial z} \tag{6}$$

where z is axial distance. This would mean that s is independent of lateral diffusivity, being the same for $D = 0$ as for $D = \infty$. Actually eqn. (6) is valid only for the case where lateral diffusivity is infinite. In that case, however, it may be easily shown that coiling the column has no effect on plate height. Later the average material flux was said to be

$$q = c^* R v_0 - \frac{7\, v_0^2 r_0^4 R}{96\, R_0^2 D} \frac{\partial c^*}{\partial z} \tag{7}$$

---

* Helicoidal coordinates would be strictly correct, but the transformations involved become very messy, and the additional precision is negligible when the pitch of the helix is much smaller than its radius as is the case in chromatographic columns.

(Note that the term for longitudinal diffusion has been omitted here, since only the increase of plate height due to coiling was of interest.) If the use of average concentrations had been correct, as was assumed in eqn. (4), then it would also be correct to write merely

$$q = c^* R v_0 \cdot \tag{8}$$

Needless to say, eqns. (7) and (8) cannot both be right, and the equivalent of (8) was used at one step in deriving (7).

A correct solution for the general case seems to be unattainable by analytic means. Certain limiting situations may be solved, however. Consider what happens when diffusivity is zero. The time needed for a bit of solute to travel a distance l along a streamline is

$$t = \frac{l}{vR} \tag{9}$$

Using eqns. (1) and (2) for l and v and letting $t_0 = l_0/v_0 R$

$$t = \left(\frac{R_0 + r \sin \theta}{R_0}\right)^2 t_0 \tag{10}$$

With the solute introduced as a Dirac function at the column inlet (*i.e.*, M moles of solute are injected instantaneously) the outlet concentration averaged over the column cross section is

$$c^* = \frac{M}{\pi F}\left[1 - \left(\frac{R_0}{r_0}\left(\frac{t}{t_0}\right)^{1/2} - \frac{R_0}{r_0}\right)^2\right]^{1/2} \frac{R_0}{r_0 t_0^{1/2} t^{1/2}}$$

where F is the volumetric flow rate of carrier gas. It is this concentration that the detector would see. This is an ellipse for very small values of r_0/R_0 and becomes skewed as $r_0/R_0 > 0.1$. A very small but finite lateral diffusivity will not alter this curve significantly. The concentration peak will break through at $t = (1 - r_0/R_0)^2 t_0$ and will end at $t = (1 + r_0/R_0)^2 t_0$. Its width w will be $4\,(r_0/R_0) t_0$. Since the number, N, of apparent transfer units is, according to KEULEMANS[2]:

$$N = \left(\frac{4\,t_0}{w}\right)^2 \tag{11}$$

we may write for the case of the coiled column

$$N = \left(\frac{R_0}{r_0}\right)^2 \tag{12}$$

The number of theoretical plates in such a case is therefore independent of column length. A column with $R_0/r_0 = 10$ would have 100 theoretical plates, clearly an undesirable situation.

Finite lateral diffusivity would tend to make the outlet peak sharper so that the very sad case of eqn. (12) would only be approximated in an ordinary packed column, although it would apply to a bundle of capillary tubes which is coiled, unless special precautions are taken to make all capillaries the same length. As lateral diffusivity approaches infinity the effect of coiling becomes very small.

Using the model of ref. 1 and the same approach as given above, the number of plates for the case of zero diffusivity would be

$$N = 4\left(\frac{R_0}{r_0}\right)^2 \tag{13}$$

and the theoretical plate height would be

$$H = l_0/N = \tfrac{1}{4}\, l_0\, (r_0/R_0)^2 \tag{14}$$

Compare this to the value obtained in ref. 1:

$$H = \frac{7\, v_0\, r_0^4}{48 R_0^2 D} \tag{15}$$

There is almost no agreement between eqns. (14) and (15).

Fig. 2 shows qualitatively the number of theoretical plates as a function of column length for various combinations of column shape and longitudinal and lateral diffusivity. The slope of curve 4 decreases as diffusivity increases. Curve 6 is tangent to 4 at length = 0 and becomes asymptotic to 3 as length increases. Curve 5 will lie between 4 and 6. Curve 2 will be between 1 and 3.

It should be kept in mind that the phenomena due to column coiling are not

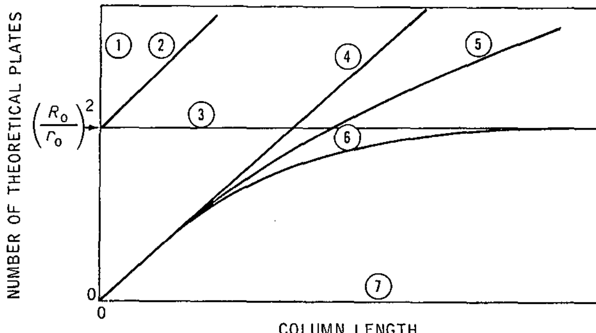

Fig. 2. Effect of diffusivity and configuration.

Curve	*Longitidinal diffusivity*	*Lateral diffusivity*	*Column shape*
1.	{ 0	any	straight
	{ 0	infinite	coiled
2.	0	finite	coiled
3.	0	0	coiled
4.	{ finite	any	straight
	{ finite	infinite	coiled
5.	finite	finite	coiled
6.	finite	0	coiled
7.	{ infinite	any	straight
	{ infinite	any	coiled

explained by analogy to a theoretical plate model. When a number of theoretical plates is assigned to a coiled column it merely refers to the amount of spreading of peaks which occurs and does not imply that a plate *mechanism* is actually applicable.

It is apparent that coiling of chromatographic column does have effects on the efficiency of the column. It seems likely, however, that an even greater loss may be caused by macroscopic disruption of the packing during coiling.

SUMMARY

The effect of column coiling upon theoretical plate height has been analyzed mathematically. A previous analysis[1] was found to employ untenable approximations. A new solution is presented for the case of zero diffusivity of solute which shows that the number of theoretical plates is:

$$N = \left(\frac{R_0}{r_0}\right)^2$$

irrespecive of column length, where R_0 is radius of the coil and r_0 is radius of the column. Qualitative relationships between column length and configuration, lateral and longitudinal diffusivity, and number of theoretical plates are also shown.

REFERENCES

1 J. C. GIDDINGS, *J. Chromatog.*, 3 (1960) 520.
2 A. I. M. KEULEMANS, *Gas Chromatography*, Reinhold, New York, 1957.

CHANGES OCCURRING WITH THE IMMOBILE LIQUID PHASE IN GAS-LIQUID CHROMATOGRAPHY

III. THE EFFECT ON OBSERVED PLATE HEIGHT

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The resolution of mixtures by gas-liquid chromatography is achieved by taking advantage of the different migration rates of the components of a mixture through a particular column under conditions which minimize zone spreading. Having once established the conditions necessary for a particular separation, it is desirable that the system be sufficiently stable that resolution is reproducible upon repetition. Failing this, it is desirable to understand the sources of instability and the manner in which they affect the resolution so that corrective measures may be taken. In the first paper of this series, KELLER *et al.*[1] reviewed the reports of the effects of instability of chromatographic columns and presented experimental data for the effect on retention volumes of liquid partitioner redistribution which occurs with repeated use of columns or with use outside of the recommended temperature. The second paper of this series[2] presented a theoretical analysis of these changes as they affect retention volumes. This third communication considers how the various forms of column instability are reflected in the zone spreading as measured by the observed plate height, H_{obs}, determined from the record of the chromatogram.

It must be stressed that the two factors of resolution, peak position and peak spreading, are basically different. The retention volume is a thermodynamic quantity which is independent of the details of the distribution of the liquid partitioner and depends upon the average amount of liquid in the column[2]. Zone spreading, however, is controlled by the kinetics of the mass transport between and within phases, and this, in turn, depends upon geometries of the phases involved. These geometries will vary over the column length as a result of column use. This variation leads to different contributions to the observed zone dimensions by the various sections along the column length. The observed plate height expression must be developed by a composition of local plate heights, H_{local}, suited to the conditions existing at particular points within the column. These equations should predict the effect of the various changes which occur with the liquid phase on the observed plate height.

THEORY

While the original theoretical model which led to the concept of the height equivalent to a theoretical plate, HETP, is no longer accepted as adequate, the utility of this parameter has not been damaged in any way. Here it is taken as a measure of zone spreading expressed as the variance or the square of the standard deviation, σ^2, of a Gaussian concentration distribution divided by the length of column traversed by the zone, L.

$$H_{\text{obs}} = \sigma^2/L \qquad (1)$$

The variance can be analyzed into a sum of terms for diffusion-like processes which depend upon the properties of the system in the region of the zone. The variance at a particular column position depends upon the residence time of the zone at that location (*i.e.*, $\sigma^2 = 2Dt$). Thus, observed plate height is a *time average* of local plate heights and measures the net amount of spreading which occurred during zone migration.

"Elution" effect

The observed plate height is routinely calculated from the variance in time of the elution diagram, τ^2, and the retention time, t_R, measured from the instant of sample injection, or some quantities equivalent to these. When the liquid partitioner has undergone macroscopic redistribution, this method of calculating plate height is not equivalent to equation (1). To distinguish it from the form of equation (1) an apparent plate height is defined as $H_{\text{app}} = L(\tau^2/t_R^2)$. The variance in time of the elution diagram is related to the zone variance in distance by the velocity of the zone at the time of elution, $u_o = R_o v_o$, where R_o is the fraction of the solute molecules in the gas phase at the column outlet and v_o is the linear gas velocity at the outlet. The latter is found by dividing the outlet gas volume rate of flow (at column temperature and outlet pressure) by the mobile phase cross-sectional area at the outlet. The retention time is related to the column length through the average zone velocity which is the product of the gas velocity and the R value averaged over the column length, $\bar{u} = \overline{Rv}$. This average must be such that $L = \bar{u}\, t_R$. The extra column contributions to retention time, *i.e.*, detector and injector designs, etc., are here taken to be negligible which, in practice, may not be true at all. The observed plate height as defined in eqn. (1) is calculated from the measured quantities, H_{app}, by the following expression:

$$H_{\text{obs}} = L\,\frac{\sigma^2}{L^2} = L\,\frac{\tau^2}{t_R^2}\,\frac{(R_o v_o)^2}{(\overline{Rv})^2} = H_{\text{app}}\,\frac{(R_o v_o)^2}{(\overline{Rv})^2} \qquad (2)$$

The factor $(R_o v_o/\overline{Rv})^2$ is generally neglected in plate height calculations. While this approximation is likely valid for cases of small pressure drop acros the column and a uniform distribution of partitioning liquid, it is not valid when the partitioning liquid is unevenly distributed along the column length. The neglect of changes in this velocity ratio with column ageing will lead to changes in the calculated plate height which are unrelated to the real efficiency of the column.

Consider two columns which are of the same length, L, and which contain the same amounts of partitioning liquid but where this liquid is uniformly distributed throughout the first column, *e.g.*, c g per unit length, in the second there is a greater

solvent load near the outlet and a smaller load near the inlet, *e.g.*, $(3/2)c$ for the last half of the column and $(1/2)c$ for the first half. In Parts I and II[1,2] of this series it was concluded that the retention times would be the same for the two columns at equal flow rates. For the sake of simplicity of the immediate argument let it be assumed that the zone spreading is directly proportional to the average partitioner load, which, in this case, is the same in both columns and that a solute zone occupies j cm of column at the outlet for both columns. We later show that this cannot be true. As a further assumption, let the inlet and outlet pressures be the same for both columns and their difference be small enough that the carrier gas velocity at the outlet, v_0, is essentially the same as the average carrier velocity, $\bar{v}$. For the first column, the solute velocity at the outlet is $u_o(1) = R_o(1)v_o$ and the zone requires $j/[R_o(1)v_o]$ seconds to emerge; for the second column $u_o(2) = R_o(2)v_o$ and the elution time is $j/[R_o(2)v_o]$. But, $u_o(2) < u_o(1)$ by virtue of $R_o(2) < R_o(1)$ because of the higher solvent load at the outlet. Hence, at the outlet, the elution times are different and one observes a broader band in terms of time (larger τ^2) for the nonuniform column and a larger H_{app} than for the uniform column since the retention times are identical. By hypothesis, this difference does not exist in H_{obs}. The argument is equally valid for plate heights based on $(1/4 \text{ peak width})^2/(\text{peak retention})^2$ as determined from distances measured on the record. Another way of expressing this result is to state that $R_o v_o/\overline{Rv} \neq 1$ for the nonuniform column even though $v_o \simeq \bar{v}$. For the first column $R_o = \bar{R}$. If one reverses the second column so that the heavier load is at the inlet, the directions of the inequalities are reversed and one ought to observe a more efficient behavior if this effect is the only one to be considered.

"R-gradient" effect

However, for the situation of macroscopic redistribution without loss, resulting in a nonuniform liquid load, the real variances of the two zones referred to in the previous section will not be the same. A positive longitudinal gradient of the cross-sectional area of partitioning liquid, *i.e.*, a greater liquid load at the outlet end, will exert a zone sharpening effect because the velocity of the front of the zone will be less than that at the tail. The difference in velocity of the front and the tail of a zone separated by one standard deviation, σ_i, may be approximated as

$$\left(\frac{\mathrm{d}R}{\mathrm{d}L}\right)_i v_i \sigma_i \tag{3}$$

where the index i indicates a particular location along the column. This expression assumes that the trans-zone zone-velocity gradient is independent of the local spreading rate and that the gradient is linear across the zone. The amount of contraction or expansion of the zone, $\delta\sigma$, is this difference multiplied by the retention time in the ith segment of the column, $\mathrm{d}L/R_i v_i$. The total change in zone width due to the R-gradient is given by the integral:

$$\Delta\sigma = \int_0^L \left(\frac{\mathrm{d}R}{\mathrm{d}L}\right) \frac{\sigma}{R}\, \mathrm{d}L \tag{4}$$

Evaluation of this integral requires a detailed knowledge of the liquid distribution.

If it is recognized that the R-gradient always has the opposite sign to the gradient in the cross-sectional area of the liquid load, A_1, then it follows that $\Delta\sigma < 0$ for a liquid distribution that increases from inlet to outlet and that $\Delta\sigma > 0$ for a liquid distribution which decreases from inlet to outlet. For the usual case of a decreased load at the inlet resulting from column bleeding, the change in zone variance arising out of the R-effect operates in the opposite sense to the change due to the elution-effect discussed earlier (eqn. 2). It will be illustrated by the experimental section that the two effects may very nearly compensate each other. This seems to argue against using nonuniform columns as a way of improving column efficiency.

If $\mathrm{d}R/\mathrm{d}L$ is constant (independent of column position), eqn. (4) can be written

$$\Delta\sigma = \sigma\int_{R_{in}}^{R_0} \frac{\mathrm{d}R}{R} = \sigma \ln \frac{R_o}{R_{in}} \tag{5}$$

where R_{in} is the R-value at the column inlet.

Now, reconsider the two columns proposed earlier. For the uniform column $\mathrm{d}R/\mathrm{d}L = 0$, so $\Delta\sigma = 0$. This calls attention to the oversimplification of the present discussion since there is always a change in band width due to diffusion processes. For the discontinuous column, where there is a sudden stepwise change of concentration, σ remains constant up to the discontinuity. At this point the front of the band changes velocity from $R_{in}v_o$ to $R_o v_o$. The tail undergoes the same change when it reaches the discontinuity. For a band of width σ the change in width is

$$\Delta\sigma = (R_o - R_{in})v_o\sigma \tag{6}$$

From this point on the band proceeds with width $(\delta + \Delta\sigma)$. The bands in the two columns do not reach the outlet with the same width. The experimentally determined partitioner distributions reported in the first paper of this series are intermediate between the extremes represented by these two columns. The apparent zone expansion discussed in relation to eqn. (2) will also have a numerical value between these same limiting values.

"Chromatodiffusion" effect

The changes which occur in the observed (time average) plate height arising from mass transfer and diffusion are far more subtle than those due to the elution- and the R-gradient effects. They have their origin in the changes in effective film thickness of columns with nonuniform load distributions. The observed variance of an eluting zone is the sum of contributions by each increment of column. Each incremental variance is corrected for the expansion of the carrier gas which occurs therein by virtue of the pressure gradient along the column.

$$\sigma^2 = \int_0^L \frac{p_i^2}{p_o^2}\,\mathrm{d}(\sigma^2) \tag{7}$$

This integral, in which p_i and p_o are zone position pressure and outlet pressure, respectively, is the sum of the variances obtained from all spreading mechanisms operating at each position and expanded by the pressure ratio between the point and the position of observation. Since it is the sum of a sum, its value is independent of the order of summation.

Considerable effort has been put forth in developing the theory of how various column parameters contribute to zone spreading. The most widely used of these expressions is the VAN DEEMTER, ZUIDERWEG AND KLINKENBERG equation[3]

$$H_{\text{local}} = 2\lambda d_p + \frac{2\gamma D_1}{p_i v_i} + \frac{8}{\pi^2} R(1 - R) v_i \frac{d_f}{D_2} \tag{8}$$

where d_p is particle diameter, λ is the eddy diffusivity factor, D_1' is molecular diffusivity of a solute in the mobile phase times the pressure at column position i, γ is the labyrinth factor, d_f is the effective film thickness, and D_2 is the molecular diffusivity of the solute in the immobile liquid phase. Rigorously, this, and related expressions, give the local plate height or the variance for an infinitesimal segment of specific geometry. Equation (9) defines the local plate height in terms of the variance for the column segment between L_i and $L_i + \text{d}L$.

$$H_{\text{local}} = \text{d}(\sigma^2)/\text{d}L \tag{9}$$

A combination of eqns. (1), (7) and (9) gives the relationship between the observed and local plate heights.

$$H_{\text{obs}} = \frac{1}{L} \int H_{\text{local}} \frac{p_i^2}{p_o^2} \text{d}L \tag{10}$$

Both the mobile phase velocity, v_i, and the local pressure, p_i, which occur in the union of eqns. (8) and (10) are non-linear functions of the column position, L_i[4]. Very little error is introduced into calculations for systems where there is a small pressure drop across the column and where v_i is assumed to be constant and $p_i/p_o = 1$.

The conditions for the application of the Van Deemter equation are that lateral diffusion, *i.e.*, into and out of the liquid normal to the carrier flow, is rate controlling and that the eddy diffusion term, $2\lambda d_p$, is independent of velocity. Substitution of the Van Deemter expression into the numerator of eqn. (10) yields a sum of three terms describing contributions to the plate height, eddy diffusion, molecular diffusion and chromatodiffusion, respectively.

$$H_{\text{obs}} = 2\lambda d_p + \frac{2\gamma D_1'}{pv} + \frac{8}{\pi^2 L} \frac{R_i(1 - R_i) v_i d_f^2}{D_2} \text{d}L \tag{11}$$

Only the last term depends upon the details of the liquid distribution. In addition to d_f, the liquid film thickness, the R_i is subject to change with loss of partitioning agent and with macroscopic redistribution. The film depth can also change when there is a microscopic redistribution.

The relationship between film thickness and cross-section is quite dependent on the geometry. For GIDDINGS' sawtooth model of the support surface[5] and for uniform impenetrable spheres, the film thickness is directly proportional to the liquid cross-section. With the approximation made so far, eqn. (11) may be written:

$$H_{\text{obs}} = S + \frac{T}{L} \int_0^L R_i(1 - R_i) d_f^2 \text{d}L \tag{12}$$

where S and T are constants made up of parameters which are not functions of L. If it is assumed that the distribution coefficient in the expression for R is constant and that macroscopic redistribution of partitioner does not alter the cross-section of the mobile phase to any significant extent, then

$$R = \frac{1}{1 + k_1 A_L} \tag{13}$$

where k_1 is a constant. In order to examine the influence of the liquid distribution on H_{obs}, assume that the column packing consists of impenetrable uniform spheres of radius r, uniformly coated with liquid to a depth d_f. GIDDINGS'[6] root-mean-square film thickness is the equivalent of this uniform film. The volume of liquid on a single particle V_p, is given by

$$V_p = \tfrac{4}{3}\pi[(r + d_f)^3 - r^3] \tag{14}$$

If this expression is expanded and only first order terms in the film thickness are retained, then

$$V_p = 4\pi r^2 d_f \tag{15}$$

If the number of particles in a unit length of column is ρ, then the volume of liquid per unit length of column, A_L, is

$$V_L = 4\pi r^2 \rho d_f = k_2 d_f = A_L \tag{16}$$

where k_2 is a constant for a uniformly packed column. Equation (13) becomes

$$R = \frac{1}{1 + kd_f} \tag{17}$$

where $k = k_1 k_2$ = constant. Equation (12) becomes

$$H_{obs} = S + \frac{Tk}{L} \int_0^L \frac{d_f{}^3}{(1 + kd_f)^2} \, dL \tag{18}$$

This equation may be used to predict the change in band width in a semiquantitative manner. In doing this, it will be assumed that S is insensitive to solute identity.

First assume a uniform partitioner distribution so that d_f is a constant. Equation (18) integrates to

$$H_{obs} = S + \frac{Tkd_f{}^2}{(1 + kd_f)^2} \tag{19}$$

For solutes which have a retention volume nearly the same as the air peak, *i.e.*, $R \doteq 1$ or $kd_f \ll 1$, then

$$H_{obs} = S + Tkd_f{}^3 \tag{20}$$

For solutes of a very large retention volume, *i.e.*, $kd_f \gg 1$

$$H_{obs} = S + \frac{T}{k} d_f \tag{21}$$

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The observed plate height is more dependent on the film thickness for very fast solute peaks than it is for very slow peaks.

For the situation of constant but different liquid distributions in two sections of the column, eqn. (18) becomes

$$H_{\text{obs}} = S + \frac{Tkd_{f1}^3}{L(1 + kd_{f1})^2}\int_0^{L_1} \mathrm{d}L + \frac{Tkd_{f2}^3}{L(1 + kd_{f2})^2}\int_{L_1}^{L} dL \tag{22}$$

where d_{f_1} is the film thickness in the region 0 to L_1 and d_{f_2} is the thickness in the region L_1 to L. Consider the hypothetical column in which half of the liquid is removed from the first half of the column and deposited in the outlet half so that in eqn. (22) the film thickness may be expressed as $d_f/2$ for the first half of the column length and as $3d_f/2$ in the outlet half.
Integration yields

$$H_{\text{obs}} = S + \frac{Tkd_f^3}{4}\left[\frac{1}{2 + kd_f)^2} + \frac{27}{(2 + 3kd_f)^2}\right] \tag{23}$$

In the limiting case of $kd_f \ll 1$

$$H_{\text{obs}} = S + {}^7/_4 Tkd_f^3 \tag{24}$$

and for $kd_f \gg 1$

$$H_{\text{obs}} = S + \frac{Td_f}{k} \tag{25}$$

Comparison of these results with those for a uniform column, eqns. (20) and (21), reveals that band broadening due to chromatodiffusion is very nearly double for fast peaks but is unaltered for very slow peaks when there is uneven distribution.

The integrals of eqn. (22) are evaluated over the lengths of the respective regions and their values are independent of the location relative to the column outlet.

It is interesting to inquire if there is a particular stepwise distribution, *i.e.*, values of the lengths of sections of column and their partitioner loads for which H_{obs} is less than that of the uniform column, or lacking this, if a maximum or minimum exists as these lengths and loads are varied. This question concerns the conditions for which the total derivative of H_{obs} vanished, *i.e.*,

$$\mathrm{d}H_{\text{obs}} = \frac{\partial H_{\text{obs}}}{\partial d_{f1}}\mathrm{d}(d_{f1}) + \frac{\partial H_{\text{obs}}}{\partial d_{f2}}\mathrm{d}(d_{f2}) + \frac{\partial H_{\text{obs}}}{\partial L_1}\mathrm{d}L_1 + \frac{\partial H_{\text{obs}}}{\partial L_2}\mathrm{d}L_2 = 0 \tag{26}$$

However, these thicknesses and lengths cannot be varied arbitrarily but must have values which satisfy the restraining conditions that there shall be no loss of partitioner from the column;

$$d_{f1}L_1 + d_{f2}L_2 = C = \text{constant} \tag{27}$$

and that the length of column is constant;

$$L_1 + L_2 = L \tag{28}$$

This problem is most easily approached by Lagrange's method of undetermined

multipliers which yields the set of six simultaneous equations in six unknowns, d_{f1}, d_{f2}, L_1, L_2 and the two undetermined multipliers μ and η.

$$\left[\frac{Tkd_{f1}^2}{L}\frac{(3 + kd_{f1})}{(1 + kd_{f1})^3} + \mu\right]L_1 = 0 \qquad (29)$$

$$\left[\frac{Tkd_{f2}^2}{L}\frac{(3 + kd_{f2})}{(1 + kd_{f2})^3} + \mu\right]L_2 = 0 \qquad (29)$$

$$\frac{Tkd_{f1}^3}{L(1 + kd_{f1})^2} + \mu d_{f1} + \eta = 0$$

$$\frac{Tkd_{f2}^3}{L(1 + kd_{f2})^2} + \mu d_{f2} + \eta = 0 \qquad (29)$$

and equations (27) and (28).

It is sufficient to eliminate μ from the first two equations which, on noting that $Tk/L \neq 0$, gives

$$L_1L_2\left[\frac{d_{f1}^2\,(3 + kd_{f1})}{(1 + kd_{f1})^3} - \frac{d_{f2}^2\,(3 + kd_{f2})}{(1 + kd_{f2})^3}\right] = 0 \qquad (30)$$

Since kd_{f1} and kd_{f2} cannot be negative so that $(3 + kd_{f1})$ and $(3 + kd_{f2})$ cannot vanish, it must be that for a maximum or a minimum either $L_1 = 0$, or $L_2 = 0$, or $d_{f1} = d_{f2}$. All of these imply a uniform liquid load. Since it has been shown that an arbitrary stepwise distribution leads to an increased H_{obs} for fast peaks, the uniform column must represent a minimum in H_{obs} and any redistribution which leads to a stepwise or discontinuous distribution must show a loss of efficiency with use. This result holds for any particular partitioner load.

In all of the discussion it has been tacitly assumed that the partitioning liquid undergoes no chemical change so that the distribution coefficient, α, as it appears in R, and the liquid diffusion coefficient, D_2, as it appears in the chromatodiffusion term of eqn. (11), undergo no change with use. This may not be true as has been pointed out previously[1,2]. Such changes have not been the subject of extensive study. One may expect that materials which polymerize further on ageing will lead to a decrease in D_2 with concomitant increase in the observed plate height. At this point it is difficult to generalize further.

EXPERIMENTAL

Two $^1/_4$ in. O.D. copper tubing columns, 1 m in length, were packed with 60–80 mesh Chromosorb P loaded with 7.5 and 15 % Apiezon L, respectively. The columns were joined in series and placed in a PE 154 unit with a thermal conductivity detector. The columns were conditioned for 4 h at 150° using He as carrier with the 15 % column at the outlet side. Four microliter samples of n-heptane were injected using the flow rates indicated by the experimental points in Fig. 1. The operating temperature was 100°. The column was reversed and the procedure repeated at otherwise identical conditions. Return of the column to the initial orientation and a repetition of the procedure gave results consistent with the initial run. Flow rates were deter-

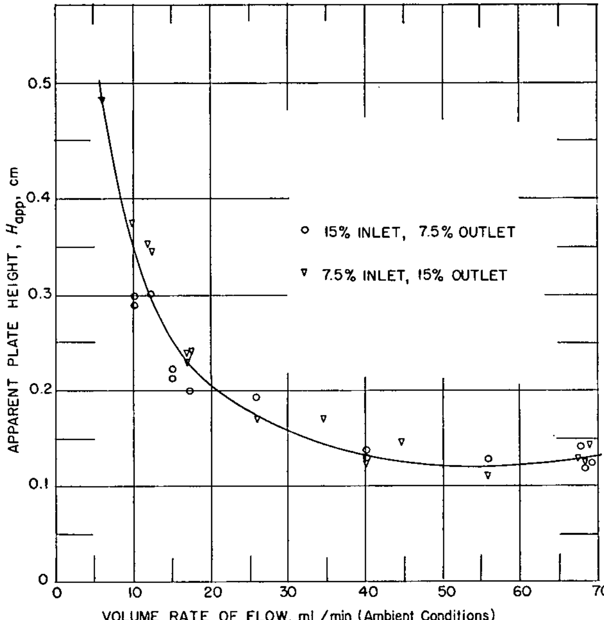

Fig. 1. The reversal of flow through a discontinuous column does not appreciably affect the apparent plate height. This illustrates the compensation of the elution effect by the R-gradient effect.

mined with a soap film flow meter. The results are shown in Fig. 1 where H_{app} is graphed *versus* the flowrate.

It is concluded that H_{app} is independent of column orientation within the precision of the experiment.

DISCUSSION

The conclusion applies only to chromatographic columns in which there is a stepwise distribution of partitioning liquid. It is assumed that the change giving rise to this redistribution occurs with no net loss of partitioner from the column, *i.e.*, columns of constant total liquid content are compared. The first condition is realistic in the sense that carrier gas which is unsaturated with partitioner will lead to evaporation of this liquid at the column inlet. Upon the carrier gas becoming saturated, evaporation will suddenly cease to give a discontinuity. That such a situation arises experimentally was shown by earlier communications in this series. The second postulate is not encountered experimentally since it assumes a volatile partitioner without column bleeding. This restriction is made in order to isolate the effect of redistribution without compounding the difficulties of a theoretical treatment. Practically, it is of interest to inquire if such a distribution will enhance column performance. The treatment presented assumes that inlet and outlet pressures are nearly equal, that the carrier gas velocity at the outlet is the same as the average velocity through the column, that there is no change in the chemical and physical properties of the partitioner, that the support is perfectly inert and does not participate in retention, that the only term in the Van Deemter equation affected by the partitioner distribution is the one describing chromatodiffusion and that the entire equation is independ-

ent of solute identity, and that a microscopic equilibrium distribution of liquid on the support exists. The model chosen for calculations presumes a packing of perfect spheres, all of the same size, uniformly coated with liquid. Examples of the contributions of various geometries of partitioning liquid and flow patterns of the mobile phase to local plate heights have been presented by GIDDINGS[6]. Nonuniform columns introduce two new effects not encountered in uniform columns. The "elution" effect arises from a partitioner load at the outlet which is different than the average load in the body of the column and produces an eluted peak with a width different from the actual zone width on the column. If this were the only difference in behavior, the theoretically important plate height defined in terms of the actual peak variance and column length, eqn. (1), is not equivalent to its experimental counterpart, the measurement of the peak variance and position on the chromatographic record. The "R-gradient" effect produces a contraction or expansion of the peak as the zone encounters regions of different liquid load. This change points out that peak width is not a function of average liquid load. The "elution" and "R-gradient" effects operate in opposite senses and tend to compensate each other. Experiments with a column with a discontinuous distribution show that these two effects compensate each other in the respect that the directions of operation of the column are indistinguishable from the apparent plate height. Such a complete compensation may not exist for continuous non-constant liquid distributions. Such continuous distributions are artificial in the sense that they do not arise from normal column operation but must be introduced with deliberate intent.

An examination of the chromatodiffusion term of the Van Deemter equation, which includes mass transfer and diffusion effects, shows that a change from a uniform distribution to a stepwise distribution will lead to an increased plate height and a less efficient column and is independent of the direction of operation of the column. This conclusion includes all of the assumptions inherent in the Van Deemter equation, mainly that diffusion through the liquid phase is completely rate controlling. If this is not true, the mobile phase may also contribute to local zone spreading. It should be noted that spreading due to diffusional processes in the mobile phase are functions of the relative velocity R, and are also subject to change as a function of redistribution and column bleeding. This matter has been ignored here.

In the normal operation of a column there will not only be a redistribution of liquid but also a net loss of this liquid. If the liquid evaporated at the inlet is not deposited at the outlet end but bleeds off of the column, the film thickness will decrease at the inlet end and remain constant at the outlet end. The column will behave as one of lower loading and it has been amply demonstrated by other investigators that the efficiency of a column will improve with diminishing loads[7]. The tendency to increased plate height brought about by redistribution is counteracted by the trend to a lower load column. It might be that the combination of these two effects leads to H_{obs} passing through a maximum during the early history of the column. This interesting hypothesis has not yet been subjected to mathematical analysis.

It may happen that in the preparation of a column, a microscopic equilibrium distribution does not exist, *i.e.*, the surface free energy is not constant over the liquid surface. During column conditioning, the liquid will redistribute until this condition exists. The liquid transport will take two forms. If liquid is thickly deposited in

some regions and too thinly in others, the liquid will spread. One might consider as a possible model for this situation a column containing excessively wet adsorbent in one part of the column and the sparsely covered support in the other part to give a discontinuous column. Conditioning would be a change toward the uniform column. The total amount of liquid in the column will remain constant and the average film thickness will remain constant. That this latter statement is true is shown by the hypothetical column where the film thickness is proportional to $(c/2)(L/2)$ in one half of the column and to $(3c/2)(L/2)$ in the remainder of the column (eqn. (16) relates film thickness directly to the volume of liquid). The average film thickness is the same as that of the companion uniform column. Conditioning would lead to a decrease in the plate height with this microscopic redistribution. This is a striking demonstration of the uselessness of considering the total amount of liquid and the average film thickness in relating plate heights to liquid distribution. Microscopic redistribution can also involve deposition of the liquid at the contact points between particles where the radius of curvature of the liquid surface is particularly small. This can only occur in the packed column during conditioning and will take place even if the partitioner is deposited on the support very slowly so that, before packing, the surface free energy of the liquid is constant over the support. According to the model proposed, the introduction of these "puddles" corresponds to the introduction of a discontinuity in the column, *i.e.*, the model would be a column where the packing carries a liquid load corresponding to the contact points in one part of the column and in the other, a liquid load corresponding to "non-contact point" load. Again, nothing need be known about the average film thickness to predict a decrease in column efficiency as liquid is transported to these contact points. The only supposition has been that a column containing regions differing dramatically in film thickness can be treated as a discontinuous column. With carefully prepared packing materials, it is probable that collection at the contact points is the principal change during microscopic redistribution. A column used before this equilibration is complete will likely show a maximum in the observed plate heights as this redistribution occurs and is followed by column bleeding.

The foregoing presentation demonstrates the difficulties involved in the evaluation of the parameters of a plate height expression in terms of observed plate height. Even in the absence of longitudinal nonuniformity of the column packing, there will always exist a pressure and velocity gradient. The low velocity extant at the inlet end of the column leads to a greater retention of zones in this region of the column and a greater contribution to the spreading by the local parameters in this end of the column. Another way of expressing this is to say that the solute does not experience in time the geometrical distribution of partitioner along the column. Calculation of the "time distribution" from the "geometrical distribution" is formidable since the relation between the two is non-linear. This is the motivation for assuming equal outlet and average carrier gas velocities in the theoretical calculations. In such a case, these two distributions are identical. A pressure gradient will magnify the effects of the loss of material at the column inlet.

Remarks pertinent to the participation of the support in retention as it depends on the liquid load have been made elsewhere[8].

SYMBOLS

A_1	cross-sectional area of the immobile liquid phase
c	concentration of partitioning liquid in the column
d_f	effective liquid film thickness; d_{s1}, d_{s2} effective film thicknesses in parts 1 and 2 of a column with a discontinuous liquid distribution
d_p	particle diameter
D	effective diffusion coefficient of the solute
$D_1' = D_1 p_i$	molecular diffusivity of the solute in the mobile phase times the pressure at column position i.
D_2	molecular diffusivity of the solute in the immobile liquid phase
H_{app}	apparent plate height
H_{local}	local plate height
H_{obs}	observed plate height
j	length of column occupied by the solute zone
k, k_1, k_2	constants
L	column length; L_1,L_2 lengths of sections of the column with different partitioner concentrations for a discontinuous liquid distribution
p	pressure
p_i	pressure at column position i
p_o	pressure at column outlet
r	radius of a support particle
R	fraction of solute molecules in the gas phase
R_i	R value at column position i
R_{in}	R value at column inlet
R_o	R value at column outlet; $R_o(1)$, $R_o(2)$: R value in parts 1 and 2 of a column with a discontinuous liquid distribution
S, T	constants independent of the column position and length
α	partition coefficient of the solute between the mobile and imobile phase
η	Lagrangian undetermined multiplier
λ	factor in eddy diffusivity
μ	Lagrangian undetermined multiplier
ρ	number of particles of packing in unit length of column
σ	standard deviation of a Gaussian distribution measured in units of length
σ_i	value of standard deviation at column position i
$\delta\sigma$	increment of standard deviation
τ	standard deviation of a Gaussian distribution measured in units of time.

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SUMMARY

This third communication of a series dealing with the effects of a nonuniform distribution of partitioning liquid in a column on gas–liquid chromatographic behavior treats relationships between the observed plate height and the plate height contributions of each section of the column. The specific distribution considered is stepwise, *i.e.*, the liquid load is assumed to be constant up to a certain position whereupon it suddenly changes to some new value which is then constant throughout the remainder of the column. The apparent plate height depends upon an "elution" effect arising from a liquid load different at the outlet from the average liquid load. The "elution" effect is opposed by an "R-gradient" effect arising from a change in the fraction of the number of solute molecules in the mobile phase with a change in the liquid load. Experiment shows that these two effects essentially cancel each other. An examination of the chromatodiffusion term of the Van Deemter equation, which deals with mass transfer and diffusion effects, shows that for a constant total amount of liquid in the column with the assumed distribution, is less efficient than one with a uniform distribution. The results are independent of the direction of carrier gas flow. The implications of these results with regard to column conditioning and use are discussed.

REFERENCES

[1] R. A. Keller, R. Bate, B. Costa and P. Forman, *J. Chromatog.*, 8 (1962) 157.
[2] R. A. Keller and G. H. Stewart, *J. Chromatog.*, 9 (1962) 1.
[3] J. J. van Deemter, F. J. Zuiderweg and A. Klinkenberg, *Chem. Eng. Sci.*, 5 (1956) 271.
[4] G. H. Stewart, S. L. Seager and J. C. Giddings, *Anal. Chem.*, 31 (1959) 1738.
[5] J. C. Giddings, *Anal. Chem.*, 34 (1962) 458.
[6] J. C. Giddings, *J. Chromatog.*, 5 (1961) 46.
[7] J. J. Duffield and L. B. Rogers, *Anal. Chem.*, 32 (1960) 340.
[8] R. A. Keller and G. H. Stewart, *Anal. Chem.*, 34 (1962) 1834.

A NOVEL TECHNIQUE FOR FILLING WIDE DIAMETER GAS CHROMATOGRAPHIC COLUMNS

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INTRODUCTION

The low efficiencies found with large diameter columns are due mainly to the method of packing[1-4]. Various methods have been devised by which efficient columns can be produced, such as beating the column while adding the packing at a controlled rate, vibrating the column when filled and pouring the packing in without vibration. These methods are, however, not very reproducible.

We have based our methods of packing large diameter columns on the fluidization technique. The columns, which can be prepared by anybody, are efficient and reproducible. The time taken is not more than 10 min for a column, 1 m long, of any diameter.

For this present study, we have used stainless steel columns, 6 cm I.D. and 1 m long.

A gauge to support the packing is placed at the bottom of the column which is then filled with the chosen size of packing without any special precautions. The packing is fluidized for a few minutes by means of a stream of nitrogen fed into the column through a conical adaptor. When a steady fluidized state has been reached the nitrogen flow rate is gradually decreased until a fixed bed is obtained; the column is then ready for use and has the properties of a fixed bed.

EXPERIMENTAL

Test have been made under the following conditions:

Column. Stainless steel; I.D.: 6 cm; length: 1 m.Filling: Chromosorb P. Particle size: 30–40 mesh. Stationary phase: silicone oil DC 200 at 20 % by weight on the Chromosorb.

Carrier gas flow rate. 250 l/h of nitrogen.

Column temperature. 40°.

Vaporizer temperature. 85°.

Sample. 1–2 ml of a 50 % mixture by weight of pentane and hexane.

RESULTS

Reproducibility of column efficiency obtained by fluidization of the packing

For every test, the column was emptied, filled and fluidized. A survey of the results is given in Table I.

For injections of hexane of 0.022 ml/cm² of column cross sectional area, the fluidization technique produces columns with an efficiency of more than 300 plates/m, with a reproducibility of over 8%.

TABLE I

Test	*Density of packing g/ml*	*Efficiency in number of theoretical plates per m of column and calculated for hexane peak*
1	0.444	313
2	0.443	330
3	0.444	324
4	0.446	322
5	0.452	302
6	0.453	303

Effect of sample injection

A 6 cm I.D. column, 1 m long, can be injected with a sample of 10 ml of pentane–hexane mixture and still give an efficiency of 80 plates/m. Fig. 1 shows the chromatogram of a 1.2 ml and a 10 ml injection. It should be noted that the pronounced tail which appears on the hexane peak for the 10 ml injection is the second impurity.

In the present work, a 10 ml injection was the maximum, since by injecting larger quantities with a syringe, the injection time becomes too long and may affect the column behaviour.

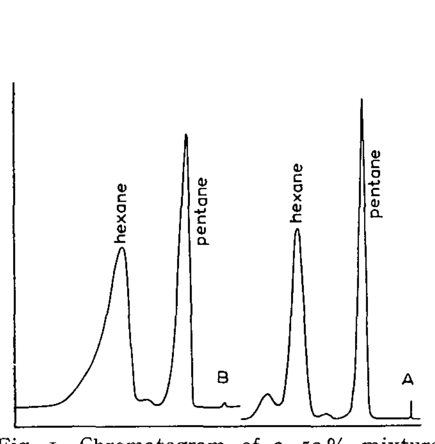

Fig. 1. Chromatogram of a 50% mixture (by weight) of pentane and hexane. Sample size: A = 1.2 ml; B = 10 ml.

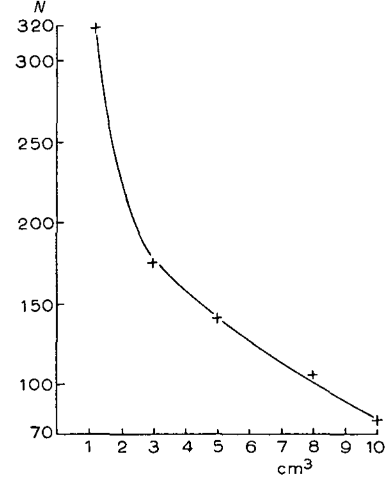

Fig. 2. Effect of sample volume on number of theoretical plates. Column length: 1 m.

Fig. 2 shows the number of theoretical plates plotted against the size of the sample injected. The curve has the appearance of part of a hyperbola.

DISCUSSION

The phenomena of fluidization have been studied by LEVA[5] and REBOUX[6]. The main properties of a fluidized bed may be summarized briefly as follows.

It is known that the pressure drop of a fluidized bed is equal to the weight of solid particles per unit cross-sectional area. Thus the pressure drop/flow diagram shows that the fluidizing point is reached at the point B corresponding to P/S in Fig. 3. With increasing fluid velocity, the pressure drop reaches a maximum and then decreases to a stable point, which has the same value as point B and is also equal to P/S.

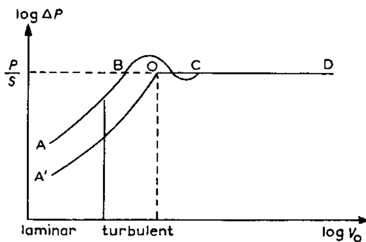

Fig. 3. Pressure drop/flow diagram according to REBOUX.

If we reverse this operation, *i.e.* start from the stable fluidized state at the point D and reduce the fluid velocity, then the pressure drop follows a new curve DOA' which has the same slope as the first but lower pressure drop values. If the fluid velocity is now increased the curve A'OD is produced but the expansion of the fixed bed occurs as soon as the value P/S is reached and there is no maximum value as in curve ABC.

The following properties distinguish an initial fixed bed and a fixed bed after fluidization:

(1) The onset of fluidization in an initial fixed bed is an unstable phenomenon and is non-reversible.

(2) The fixed bed after fluidization has reproducible reversible properties. The voidage *Em,* required for the onset of fluidization is termed the "minimum fluid voidage" and its value is higher than for an ordinary fixed bed—in this state the particles are touching.

The consequences of such a theoretically perfect filling for large diameter, preparative gas chromatographic columns are as follows:

(a) Because of the uniform compactness, the carrier gas profile inside the column must show a minimum concavity from the walls to the centre.

(b) The minimum compactness of the packing increases the radial diffusion. This property has a direct effect on the constant A of the Van Deemter equation[7]. The constant A depends upon the packing of the column and its value is $A = 2\,\lambda\,dp$, where dp is the particle diameter, and λ the measure of packing irregularities.

(c) The time taken to fill a column is definitely reduced and, in addition, the long time required to reach the desired temperature, due to the temperature gradient between the walls and the centre of the column, may be reduced. In a fluidized bed the heat transfer is at a maximum because of the large heat exchange area between the gas phase and the solid, and the turbulence of the gas and the particles. Since the temperature inside the fluidized bed is homogeneous, the preparation of the column may also be improved by fluidizing the column inside the chromatograph with carrier gas preheated to the required temperature.

CONCLUSION

The technique of fluidization has many advantages for preparative columns and provides an answer to the problem of preparing large diameter columns.

The advantages are:

(i) Preparation of the column is independent of the operator's skill.
(ii) The technique involved is very rapid.
(iii) The columns thus prepared are efficient.
(iv) The efficiency of such columns is reproducible.
(v) Finally, the column may be brought into operation more quickly by fluidizing the packing with carrier gas preheated to the required temperature.

If the technique of fluidization is applied to columns of still larger diameter, it is possible to foresee the use of preparative gas chromatography on a more important scale, perhaps reaching the size of semi-industrial separation equipment.

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SUMMARY

The fluidization technique provides a solution to the problem of filling wide diameter columns used in preparative gas chromatography.

The density of a packing after fluidization is uniform both in the direction of the column and across the diameter. When applied to preparative gas chromatographic columns, this feature of fluidization offers the possibility of obtaining a gas profile with the minimum of concavity.

The average efficiency of a 6 cm I.D. column filled in this way is 315 plates per metre when 1.2 ml of a mixture of equal parts by weight of pentane and hexane is injected.

The preparation of the columns is quick and reproducible with a performance independent of the operator.

Since with a fluidized bed, heat transfer is at a maximum, the setting up of a column can be made more rapid by using carrier gas heated to the desired temperature for fluidizing the packing.

REFERENCES

1 F. H. HUYTEN, W. VAN BEERSUM AND G. V. A. RIJNDERS, in R. P. W. SCOTT (Editor, *Gas Chromatography 1960*, Butterworths, London 1960, p. 224.
2 E. BAYER, *Angew. Chem.*, 73 (1961) 525.
3 G. J. FRISONE, *J. Chromatog.*, 6 (1961) 97.
4 P. V. PEURIFOY, J. L. OGILVIE AND I. DVORETSKY, *J. Chromatog.*, 5 (1961) 418.
5 M. LEVA, *Fluidization*, McGraw-Hill, New York, 1959.
P. REBOUX, *Phénomènes de Fluidisation*, Association Française de Fluidisation, Paris, 1954.
A. I. M. KEULEMANS, *Gas Chromatography*, 2nd Ed., Reinhold, New York, 1959.

PREPARATION OF A COMPOUND WHICH MAY BE HYDROGEN ISOCYANIDE

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The possibility of the gas chromatographic identification of the long-postulated compound hydrogen isocyanide** has turned up as an interesting side line in a recent study of the pyrolyzates of the substituted barbituric acids. Though the primary focus of the investigation was the applicability of pyrolytic gas chromatography to a toxicological problem of forensic importance, the repeated appearance of an unknown peak was felt to justify at least a limited detour into an old problem of pure chemistry.

During the preparation of hydrogen cyanide as a standard for the determination of its retention time, it was found that a substance in the vapor above the sample of dry KCN had a retention time which differed from that of HCN prepared by adding acid to KCN. In a 4-ft. column packed with polypropylene glycol 10 % on celite 100/120 the retention times in minutes, at a flow rate of 50 ml/min, were:

Temperature °C	*HCN*	*Unknown*
50	4.6	6.6
75	2.15	3.2

Both HCN and the unknown vapor were unusual in that they produced negative peaks with the ^{90}Sr radioactive detector in the Pye gas chromatograph. The peaks were reasonably sharp and slightly asymmetrical. Substances which give negative peaks with the ^{90}Sr ionization detector when argon is the carrier gas are comparatively few in number. They are substances whose ionization potential is greater than the excitation potential of the argon atoms (11.6 eV), *viz.*:

Br_2	H_2	O_2	CO_2	HCN	H_2O
Cl_2	Kr	Xe	CF_4	HCl	N_2O
F_2	Ne	BF_3	$(CN)_2$	HF	SO_2
He	N_2	CO	HBr	HI	SF_6

Methane, C_2H_4 and C_2H_6, though they fall into this category so far as their ionization potentials are concerned, do, however, ionize sufficiently to give positive peaks.

* On leave, at the time of this investigation, from the Dominion Laboratory, Auckland, New Zealand.
** Alternative names: hydrogen isonitrile, hydrogen carbylamine.

Water gives a negative peak at low temperatures and a positive peak over 100°. Most organic compounds have molecules whose ionization potentials are less than the excitation potential of the argon atoms, and hence give positive peaks.

Hydrogen cyanide is believed to exist in two forms because it gives rise to two kinds of alkyl derivatives: the alkyl cyanides, RCN, which are derivatives of hydrogen cyanide, HCN, and the alkyl isocyanides, RNC, which are derivatives of hydrogen isocyanide, HNC.

EARLIER APPROACHES TO THE PREPARATION OF HNC

USHERWOOD[1] calculated from the ratio of specific heats of gaseous HCN that the equilibrium mixture contains a few tenths of 1 % of HNC. ENKLAAR[2] claimed to have isolated the isomerides of which HCN was thought to consist by means of their methyl mercuric salts. He claimed that the isocyanide could be prepared by the action of H_2S on a solution of AgCN, of $Hg(CN)_2$ or of AgCN in KCN, or by the action of H_2SO_4 on $Na_4Fe(CN)_6$. He later[3] attempted to calculate the relative proportions of the isomers in ordinary prussic acid from boiling-point data. COATES, HINKEL AND ANGEL[4] attempted to repeat ENKLAAR'S work with mercuric methyl cyanides. All of their preparations gave the usual mercuric methyl cyanide, and they found no evidence for its alleged isomer.

DADIEU[5] from a study of the Raman spectrum of HCN deduced evidence for the presence of two types of molecules in the gaseous state, but the amount of hydrogen isocyanide was of the order of 0.5 % or under. GORDY AND WILLIAMS[6] examined the infrared spectrum of aqueous solutions of HCN and deduced that a weak band was due to 2–4 % of HNC. From a similar study of the Raman spectrum HERZBERG[7] found no evidence to support the view that traces of HNC were present in HCN. REICHEL AND STRASSER[8] studied the ultraviolet curve of HCN and concluded that at most it contains only a few parts per hundred of the isomer.

The alkyl and aryl isocyanides form isothiocyanates by direct union with sulfur. However, efforts made by MCCROSKY, BERGSTROM AND WAITKINS[9] to parallel this synthesis and form isothiocyanic acid from hydrogen isocyanide (presumed to be present in HCN) resulted in failure. They concluded that HNC cannot be present in pure HCN except in extremely small concentrations.

ESCUBÓS, VARGAS, AND SOLS[10] argue the presence of HNC in HCN from its inhibiting action on phosphomonoesterases.

More recently a group of chemists at Cambridge University[11], approaching the problem from the theoretical side, have concluded that under mild conditions the preparation of hydrogen isocyanide should be possible. Their conclusion was based upon the fact that the energy potential of a proton brought into juxtaposition with the C–N group shows a minimum in the position appropriate to HCN and another, somewhat higher, corresponding to HNC.

TWO APPROACHES USED IN THIS STUDY

The unknown compound isolated gas-chromatographically in this study has been investigated by (a) excluding possible known compounds and (b) attempting to prepare it by alternative routes.

Survey of possible known compounds

Cyanogen, $(CN)_2$, was prepared by adding a solution of KCN to a solution of $CuSO_4$. The retention time at 50° was 0.75 min; negative peak.

Cyanic acid, HOCN, was prepared by dry distillation of cyanuric acid. It gave a very flat, extremely asymmetrical positive peak with retention times at 75° varying from 7.5 to 15 min, depending upon the amount injected.

Hydrazine, $H_2N \cdot NH_2$. Attempts were made to prepare hydrazine from the sulfate with strong potassium hydroxide. Two positive peaks were obtained from the vapor; the retention times were 2.8 min and 6.5 min.

Ammonia, NH_3; positive peak at 1.55 min.

Hydroxylamine, $HO \cdot NH_2$. Attempts were made to prepare hydroxylamine by adding alkali (dil. KOH, conc. KOH and moist $Ca(OH)_2$) to the hydrochloride. In each case the vapor gave a small positive peak at 1.0 min. It is reported to be unstable and to decompose rapidly at room temperature, especially in the presence of moisture and CO_2.

Attempts to prepare hydrogen isocyanide

1. One of the general methods for preparing isocyanides is by heating an alkyl iodide with silver cyanide in aqueous ethanolic solution.:

$$RI + AgCN \longrightarrow R \cdot NC + AgI$$

A small amount of alkyl cyanide is also formed. On the other hand, if KCN is used, the major product is cyanide and traces only of isocyanide are obtained[12].

If HI had been heated with AgCN in aqueous ethanolic solution, any possible peak due to hydrogen isocyanide would have been obscured by the positive ethanol peak if the polypropylene glycol column was used. AgCN reacted rapidly with hydriodic acid (in the absence of ethanol) but the product appeared to be mainly HCN.

When AgCN was reacted with HCl, either dilute or concentrated, no negative peak at 3.2 min (75°) was observed. However, when dry hydrogen chloride was reacted with dry AgCN the resulting vapor produced a small negative peak at 3.2 min.

2. Heating a mixture of a primary amine and chloroform with ethanolic potassium hydroxide is a method for preparing isocyanides and is also the well-known carbylamine test for a primary amine.

$$R \cdot NH_2 + CHCl_3 + 3KOH \longrightarrow R \cdot NC + 3KCl + 3H_2O$$

FINAR[12] points out that it has been argued by analogy that using ammonia as the primary amine might lead to the formation of hydrogen isocyanide.

When a small amount of chloroform was warmed with ammonia and potassium hydroxide, some samples of the vapor in the test tube produced small negative peaks. The negative peak corresponding with the presumed hydrogen isocyanide was variable in relative size from experiment to experiment, and was frequently absent. The optimum conditions for this preparation have not yet been determined.

3. Isocyanides may be prepared by reacting N-substituted formamides with $POCl_3$[13,14].

$$HCON\begin{matrix}\diagup R \\ \diagdown H\end{matrix} \xrightarrow[(-H_2O)]{POCl_3} CN\text{-}R$$

On the other hand, alkyl cyanides may be prepared by treating acid amides with P_2O_5 or $POCl_3$. Formamide might, therefore, give either HCN or HNC when it reacts with P_2O_5 or $POCl_3$.

When formamide was treated with P_2O_5, either cold or with heating, the product was HCN in which none of the substance sought could be detected. However, when $POCl_3$ was used, the chromatograph indicated that there was a significant amount of the supposed isocyanide present in the products, which consisted mainly of HCN. In test-tube experiments with $POCl_3$ in excess, the vapors contained HCN and the presumed hydrogen isocyanide in a ratio of 10 or 20 to 1. When, however, formamide was in excess, the ratio of HCN to the supposed HNC was about 2 or 3 to 1. Attempts were made to scale up this preparation, but the resulting product appeared to be mainly HCN in which only trace amounts of the substance sought could be detected with the gas chromatograph. The reaction of formamide with $POCl_3$ is exothermic, and the heat so produced may depress yields of the supposed isocyanide.

HCN prepared by treating KCN with dilute sulfuric acid contained no trace of hydrogen isocyanide. Attempts to prepare HNC by treating potassium ferrocyanide or potassium silver cyanide complexes with acid have also been unsuccessful up to the present.

Although the existence of hydrogen isocyanide has not yet been proved, there is enough evidence here to justify further investigation.

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SUMMARY

To hydrogen isocyanide, long-postulated isomer of hydrogen cyanide, may be attributed a small negative peak which was observed with a ^{90}Sr detector. Tentative partial identification by synthesis of the compound is here reported. The peak appeared repeatedly in a study of the pyrolytic gas chromatography of the substituted barbituric acids.

REFERENCES

1 E. H. USHERWOOD, *J. Chem. Soc.*, 121, (1922) 1604.
2 C. J. ENKLAAR, *Rec. Trav. Chim.*, 44 (1925) 889; *C.A.*, 20 (1926) 582; *J. Chem. Soc.*, 128 (1925) A I 1394.
3 C. J. ENKLAAR, *Rec. Trav. Chim.*, 45 (1926) 414; *C.A.*, 20 (1926) 2729; *Rec. Trav. Chim.*, 46 (1927) 709; *C.A.*, 22 (1928) 191.
4 J. E. COATES, L. E. HINKEL AND T. H. ANGEL, *J. Chem. Soc.*, (1928) 540.
5 A. DADIEU, *Ber.*, 64B (1931) 358; *C.A.*, 25 (1931) 2364; *Monatsh.*, 57 (1931) 437; *C.A.*, 25 (1931) 4795.
6 W. GORDY AND D. WILLIAMS, *J. Chem. Phys.*, 4 (1936) 85.
7 G. HERZBERG, *J. Chem. Phys.*, 8 (1940) 847.
8 L. REICHEL AND O. STRASSER, *Ber.*, 64B (1931) 1997; *C.A.*, 25 (1931) 5893.
9 C. R. MCCROSKY, F. W. BERGSTROM AND G. WAITKINS, *J. Am. Chem. Soc.*, 64 (1942) 722.
10 J. M. ESCUBÓS, J. J. VARGAS AND A. SOLS, *Rev. Españ. Fisiol.*, 3 (1947) 281; *C.A.*, 42 (1948) 7804.
11 S. F. BOYS, Cambridge University, England, personal communication.
12 I. L. FINAR, *Organic Chemistry*, 3rd Ed., Longmans, Green and Co., London, New York, Toronto, 1959, Vol. I, p. 280.
13 I. UGI AND R. MEYR, *Angew. Chem.*, 70 (1958) 702; *C.A.*, 53 (1959) 10084e.
14 W. R. HERTLER AND E. J. COREY, *J. Org. Chem.*, 23 (1958) 1221.

A GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF THE ANTIOXIDANTS BHA, BHT AND ETHOXYQUIN IN AQUEOUS AND IN HYDROCARBON SOLUBLE SAMPLES

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A gas–liquid partition chromatographic (GLPC) method for the separation and determination of the antioxidants BHA [2(and 3)-*tert.*-butyl-4-hydroxyanisole], BHT (3,5-di-*tert.*-butyl-4-hydroxytoluene), and ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) in aqueous and in hydrocarbon-soluble samples is described.

BUTTERY AND STUCKEY[1] described a GLPC method for the determination of BHA and BHT in samples of potato granules. The Apiezon L column used in this method, however, must be "aged" for one week in order for the BHA determination to be satisfactory. In addition, because of the high temperatures needed, it is believed that some of the BHA was decomposed on the column, which greatly reduced its life time. Another drawback that limits the sensitivity of the determination is the base line noise observed when this column is used. The method of JENNINGS, CURRAN AND EDWARDS[2] is able to determine BHT in paperboard. The column used for this determination consisted of a propylene glycol stationary phase on C-22 Firebrick. Although this column gives satisfactory results for the determination of the antioxidant BHT in paper board, it lacks the ability necessary to separate BHT from BHA in food products. The usefulness of this column is also limited by the fact that it is specific for BHT.

Very recently SCHWECKE AND NELSON[3] described a GLPC method for the determination of BHA and BHT in samples of foods, fats, oils, and potato products. The chromatographic column used for this work consisted of a mixture of Silicone Gum SE-30 and Tween 80. Because of the use of Tween 80 the column temperature is limited to a maximum of 150°. The method also requires the need of an internal standard (di-BHA) in order to effect the quantitative determination of the two antioxidants.

The development of the simple, accurate and rapid GLPC method described here was undertaken in order to effect the simultaneous separation of the antioxidants BHA, BHT, and ethoxyquin and to determine their concentration with a sensitivity to the nearest part per million as required in food analysis. The column found to be most satisfactory to effect the separation and determination of mixtures of these antioxidants consists of a 20 weight % Silicone Gum SE-30 (General Electric Co.) on 60/80 mesh solid support of either Firebrick (Johns-Manville Co.) or Chromosorb W (Wilkens Instrument and Research, Inc.). When Firebrick was used the most effect-

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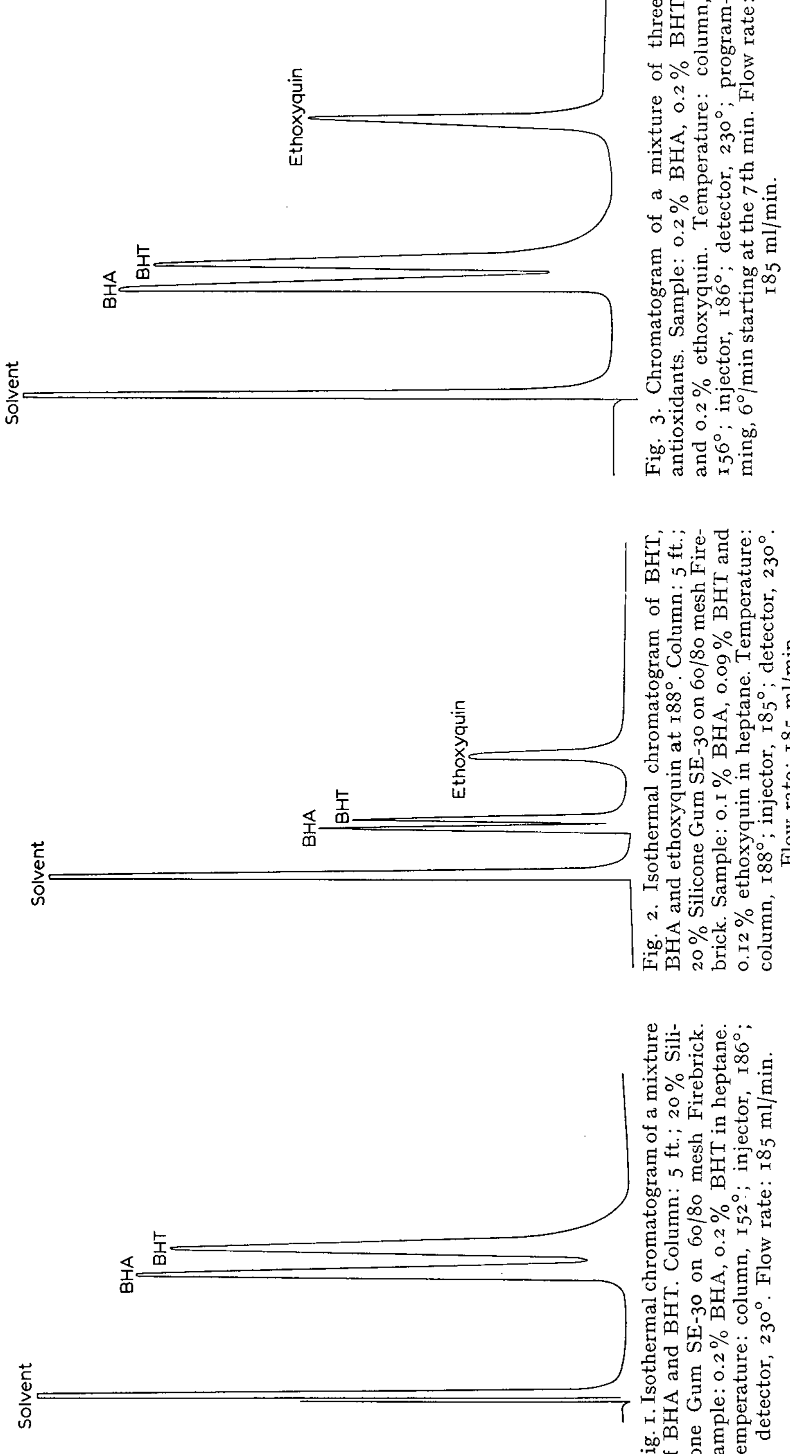

Fig. 1. Isothermal chromatogram of a mixture of BHA and BHT. Column: 5 ft.; 20% Silicone Gum SE-30 on 60/80 mesh Firebrick. Sample: 0.2% BHA, 0.2% BHT in heptane. Temperature: column, 152°; injector, 186°; detector, 230°. Flow rate: 185 ml/min.

Fig. 2. Isothermal chromatogram of BHT, BHA and ethoxyquin at 188°. Column: 5 ft.; 20% Silicone Gum SE-30 on 60/80 mesh Firebrick. Sample: 0.1% BHA, 0.09% BHT and 0.12% ethoxyquin in heptane. Temperature: column, 188°; injector, 185°; detector, 230°. Flow rate: 185 ml/min.

Fig. 3. Chromatogram of a mixture of three antioxidants. Sample: 0.2% BHA, 0.2% BHT and 0.2% ethoxyquin. Temperature: column, 156°; injector, 186°; detector, 230°; programming, 6°/min starting at the 7th min. Flow rate: 185 ml/min.

ive column length was found to be 5 ft. Helium was used as a carrier gas at various flow rates as indicated in the sample chromatograms. A 5-ft. Firebrick column operated at a column temperature of 150° was found to give the most satisfactory separation of BHA and BHT as shown in Fig. 1. For the simultaneous separation and determination of BHA, BHT, and ethoxyquin, however, a column temperature of 190° was used. An example chromatogram of this separation and determination is shown in Fig. 2. The conditions used to effect the separation of these antioxidants using a 5-ft. Firebrick column by a temperature programming procedure is indicated in the example chromatogram shown in Fig. 3.

When Chromosorb W is used as the solid support, higher weight % of Silicone Gum SE-30 or a longer column is required for the separation of BHA and BHT. In this study a 10-ft. column coated with 20 weight % of the Silicone Gum was used. The Chromosorb W column gives a clear separation of individual components and can be used for trace analysis. Figs. 4 and 5 show the difference in the detector response for the two different columns when the chromatograph was operated under identical conditions. Fig. 6 is a sample chromatogram showing the optimum conditions necessary for the simultaneous separation and determination of BHA, BHT, and ethoxyquin when a 10-ft. Chromosorb W column was used.

Purity determination of BHA, BHT, and ethoxyquin commercial samples obtained according to the conditions given in Figs. 1 and 2, are compared with the results

TABLE I

PURITY DETERMINATION OF COMMERCIAL ANTIOXIDANTS

	Purity (%)							
Sample	*BHA*		*Sample*	*BHT*		*Sample*	*Ethoxyquin*	
	*GLPC**	*Chemical***		*GLPC**	*Chemical***		*GLPC****	*Chemical***
A	99.5	99.4	D	99.4	99.2	G	98.6	98.5
	99.2	99.6		99.2	99 1		98.2	98.4
	99.7	99.5		99.5	99.0		98.0	98.2
B	99.1	99.0	E	99.5	99.3	H	97.5	97.3
	99.4	99.2		99.2	99.4		97.2	97.0
	99.6	99.1		99.7	99.2		96.9	97.1
C	98.7	98.6	F	98.2	98.4	I	97.2	97.4
	98.8	98 7		98.6	98.3		97.6	97.6
	98.4	98.5		98.7	98.6		97.5	97.7

* GLPC method shown in Fig. 1.
** Ultraviolet spectrophotometric methods.
*** GLPC method as shown in Fig. 2.

obtained by ultraviolet spectrophotometric methods[4, 5]. The results are tabulated in Table I. Using the conditions for the simultaneous separation as shown in Figs. 2, 3 and 6, the % recovery of the antioxidants BHA, BHT, and ethoxyquin in several commercial samples are compared and given in Table II.

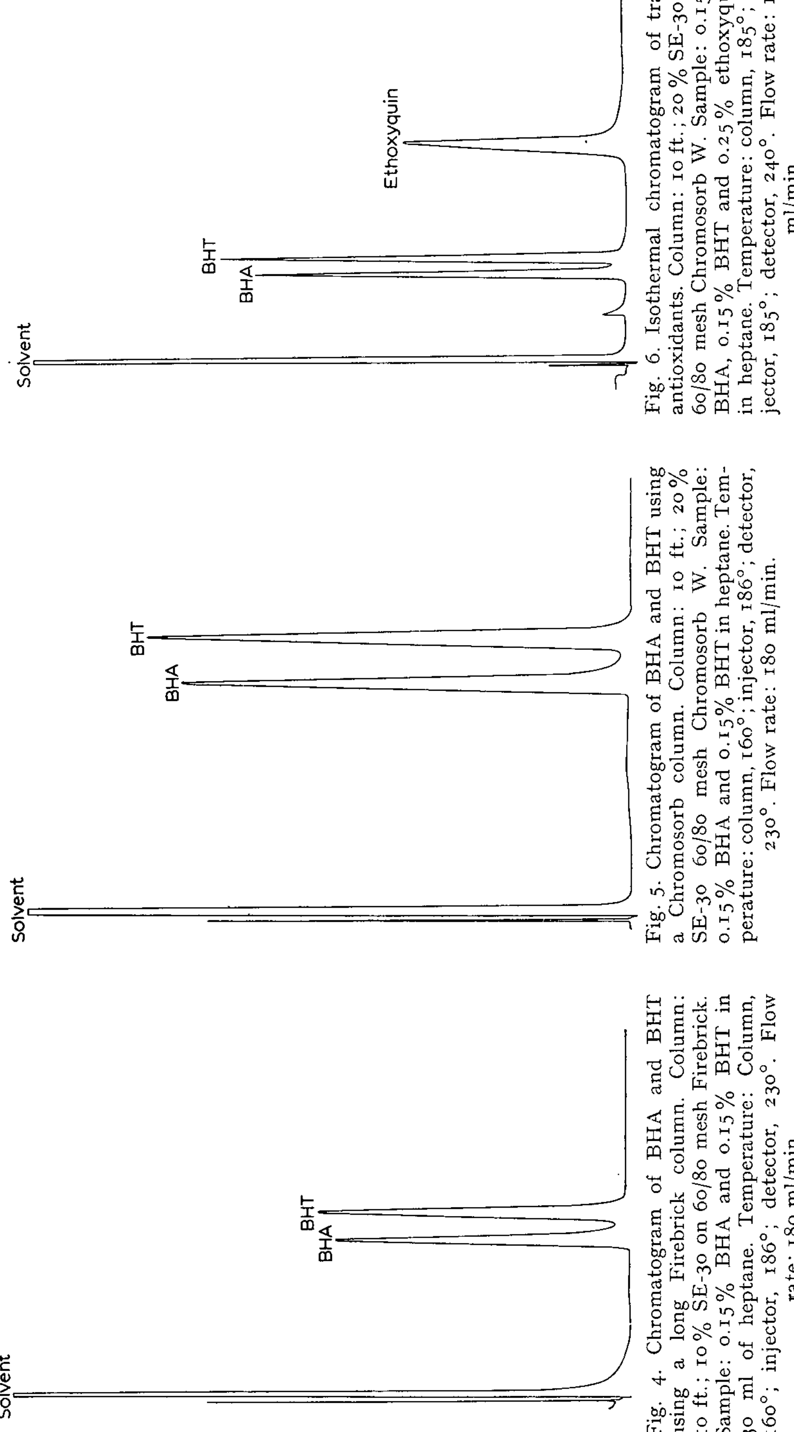

Fig. 4. Chromatogram of BHA and BHT using a long Firebrick column. Column: 10 ft.; 10% SE-30 on 60/80 mesh Firebrick. Sample: 0.15% BHA and 0.15% BHT in 30 ml of heptane. Temperature: Column, 160°; injector, 186°; detector, 230°. Flow rate: 180 ml/min.

Fig. 5. Chromatogram of BHA and BHT using a Chromosorb column. Column: 10 ft.; 20% SE-30 60/80 mesh Chromosorb W. Sample: 0.15% BHA and 0.15% BHT in heptane. Temperature: column, 160°; injector, 186°; detector, 230°. Flow rate: 180 ml/min.

Fig. 6. Isothermal chromatogram of trace antioxidants. Column: 10 ft.; 20% SE-30 on 60/80 mesh Chromosorb W. Sample: 0.15% BHA, 0.15% BHT and 0.25% ethoxyquin in heptane. Temperature: column, 185°; injector, 185°; detector, 240°. Flow rate: 175 ml/min.

TABLE II

COMPARISON OF THE SIMULTANEOUS DETERMINATION OF BHT, BHA, AND ETHOXYQUIN ON VARIOUS OPERATION CONDITIONS (mg/ml)

Sample		*BHA*	*BHT*	*Ethoxyquin*
1	Actual	0.0010	0.0010	0.0020
	Found a*	0.0009_8	0.0009_9	0.0019_7
	b	0.0009_6	0.0009_6	0.0019_4
	c	0.0010_1	0.0009_9	0.0019_6
2	Actual	0.0050	0.0050	0.0050
	Found a	0.0049	0.0050	0.0049
	b	0.0048	0.0049	0.0050
	c	0.0050	0.0050	0.0049
3	Actual	0.0125	0.0125	0.0125
	Found a	0.0123	0.0124	0.0150
	b	0.0125	0.0124	0.0148
	c	0.0125	0.0125	0.0149
4	Actual	0.250	0.250	0.300
	Found a	0.251	0.249	0.299
	b	0.248	0.251	0.297
	c	0.249	0.252	0.296

* a: conditions shown in Fig. 1 (BHA and BHT) and Fig. 2 (ethoxyquin); b: conditions shown in Fig. 3; c: conditions shown in Fig. 6. All results are averages of duplicate determinations.

EXPERIMENTAL

Instruments

An Aerograph 350-B chromatograph (Wilkens Instrument, Walnut Creek, Calif., U.S.A.) provided with dual thermal conductivity detector cells was used for our initial investigation. An Aerograph 600-B provided with a flame ionization detector was used for the trace analysis.

Carrier gas

Helium, Matheson's 1 A.

GLPC columns

The standard procedure of packing the columns was used. The Silicone Gum SE-30 was first dissolved in chloroform. A slurry of this solution with the solid support (Chromosorb W or Firebrick) was made and then dried in a vacuum oven. The actual weight % of the substrate was calculated on the dried weight basis. The column material was then packed, according to the usual procedure, into 1/4 in. and 1/8 in. copper tubing.

Materials

All reagents used were reagent grade and obtained from Eastman Chemical Products, Inc., Kingsport, Tenn., U.S.A. The standard antioxidants BHA, BHT, and ethoxyquin used in the initial investigation were freshly synthesized by Nopco Chemical Company, Organic Chemical Division, Harrison, N.J., U.S.A.

TABLE III

DETERMINATION OF ANTIOXIDANTS IN COMMERCIAL PRODUCTS (mg/g)

	BHA		*BHT*		*Ethoxyquin*	
	Claim	*Found*	*Claim*	*Found*	*Claim*	*Found*
Vitamin A oil	2.50	2.42 2.36 2.39	2.50	2.48 2.44 2.47	—	— — —
Vitamin A oil	5.00	4.88 4.92 4.87	5.00	4.86 4.92 4.90	—	— — —
Multiple vitamin mixture A	0.25	0.235 0.237 0.234	0.25	0.238 0.240 0.235	—	— — —
Multiple vitamin mixture B	1.00	0.975 0.978 0.977	1.00	0.980 0.985 0.983	1.00	0.965 0.968 0.971
Feed premix, Nopco L	—	— — —	3.50	3.42 3.40 3.39	3.50	3.44 3.38 3.36
Feed premix, Nopco V	2.28	2.24 2.25 2.26	2.85	2.78 2.76 2.78	2.65	2.61 2.59 2.60
Feed, synthetic (I)	100 p.p.m.	98.8 99.0 99.1	100 p.p.m.	99.2 99.4 99.2	100 p.p.m.	98.4 98.6 98.2
Feed, synthetic (II)	50 p.p.m.	48.5 48.7 48.8	50 p.p.m.	49.0 48.8 48.7	50 p.p.m.	48.5 48.2 48.0

PROCEDURE

Hydrocarbon-soluble samples

The sample, (fat, oil, etc.) was dissolved in a suitable low boiling point hydrocarbon. When the antioxidants were present only in trace amounts the solution was concentrated over a steam bath. No appreciable loss of antioxidants was observed due to this concentration process. Materials usually found in samples of this kind such as vitamins A, D, B_{12} and niacin, calcium *d*-pantothenate, riboflavin, etc. were found not to interfere.

Water-soluble samples

For water-soluble samples not containing proteins, direct injections of the solution into the chromatograph provided with a flame-ionization detector was found to give satisfactory results. No prior concentration of the solution was necessary and the usual ingredients found in this class of samples does not interfere with the determination.

For water-soluble samples containing proteins such as gelatin products, direct

injection of the aqueous solution was found to give rise to base line noise after several applications. For this class of samples it was found advantageous to reflux the sample with methanol, and use the methanol solution for the GLPC determination.

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SUMMARY

A gas-liquid partition chromatographic (GLPC) method for the simultaneous separation and determination, to the nearest part per million, of the antioxidants BHA, BHT, and ethoxyquin is described. The columns used to effect the separation and determination of these antioxidants consist of Silicone Gum SE-30 on either Chromosorb W or Firebrick. The relative merit of these two solid supports is indicated. The method was then applied to the determination of these antioxidants in water- and hydrocarbon-soluble commercial samples. The data obtained from the GLPC method is compared with that obtained from ultraviolet spectrophotometric studies.

REFERENCES

1 R. G. BUTTERY AND B. N. STUCKEY, *J. Agr. Food Chem.*, 9 (1961) 283.
2 E. C. JENNINGS, T. D. CURRAN AND D. G. EDWARDS, *Anal. Chem.*, 30 (1958) 1946.
3 W. M. SCHWECKE AND J. H. NELSON, *Paper presented at the 1962 Am. Chem. Soc. Fall Meeting*, Atlantic City, New Jersey.
4 N. J. ALICINO, H. C. KLEIN, J. J. QUATTRONE, JR. AND T. K. CHOY, *J. Agr. Food Chem.*, in the press.
5 T. K. CHOY, N. J. ALICINO, H. C. KLEIN AND J. J. QUATTRONE, JR., *J. Agr. Food Chem.*, 11 (1963) 340.

DETERMINATION OF SOME MIXED PHENOLIC ANTIOXIDANTS IN POLYETHYLENE

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The applications of polyethylene have increased enormously in the last decade. Typical of these new uses has been the wrapping of foods with polyethylene film. Small amounts of antioxidants are usually added to protect this plastic against deterioration during processing and to improve its aging characteristics. The most common are phenolic compounds: 4,4'-butylidene-bis-(6-*tert.*-butyl-*m*-cresol) (Santowhite Powder®)*, 2,6-di-*tert.*-butyl-*p*-cresol (BHT), and 4,4'-thio-bis-(6-*tert.*-butyl-*m*-cresol) (Santonox R®). Often these are used in combinations containing Santonox–BHT and Santowhite Powder–BHT. However, before these antioxidants could be utilized to stabilize polyethylene film for wrapping food, methods were required for determining these mixed phenolic antioxidants in polyethylene.

A number of satisfactory methods have been developed for the analyses of polyethylene containing single antioxidants. WADELIN[1] reported a method for the analysis of BHT in polyethylene based on measuring the U.V. absorbance of the potassium salt of BHT in absolute ethanol. HILTON[2] analyzed a number of antioxidants by diazo dye formation. More recently, STAFFORD[3] developed a sensitive spectrophotometric method based on the controlled oxidation of BHT. These methods are not suitable for the direct analyses of mixed antioxidants in polyethylene.

SPELL AND EDDY[4] developed a spectrophotometric method for the analyses of Santonox R and BHT based on the removal of Santonox R by basic extraction and the subsequent U.V. measurement of the separated antioxidants. However, the technique is not applicable to the separation of Santowhite Powder from BHT because Santowhite Powder is too weakly acidic.

The methods described herein involve (1) extraction of the antioxidants from the polyethylene sample, (2) separation of the extracted antioxidants on an alumina chromatographic column and (3) determination of the separated antioxidants by ultraviolet spectroscopy.

EXPERIMENTAL

Reagent and apparatus

1. Aluminum oxide (Merck Reagent grade, Catalog No. 71707) was dried at 120° under 200 mm pressure in a vacuum oven for 20 h.

2. The chloroform and methanol were A.R. grade.

3. The 10% (v/v) water in methanol reagent was prepared by adding 100 ml of distilled water to 900 ml of methanol.

* ® = trade-mark of Monsanto Chemical Company.

4. Spectrophotometric measurements were made with matched 1 cm silica cells using a Cary Model 11 Recording Spectrophotometer.

5. The chromatographic effluents were monitored by a Gilson Medical Electronics U.V. Scanner (Middleton, Wisconsin) coupled to a Varian Recorder.

Method

1. Sample preparation. If the sample of polyethylene is thicker than approximately $^1/_{16}$ in., it should be thinned by passing it through a conventional rubber or plastics mill. Weigh approximately 2.5 g of a sample containing 0.01 % to 0.3 % of phenolic antioxidant. Dice the sample into small squares (*ca.* 5 mm × 5 mm) and transfer to a bottle with a teflon lined cap. Add 50 ml of chloroform to the sample. If the sample is suspected of having less than 0.02 % antioxidant, add 25 ml of chloroform instead of 50 ml. Place the tightly capped bottle in a 50° oven and let stand for 3 h with intermittent shaking at intervals of approximately 15 min. Remove the sample and cool to ambient temperature.

2. Separation and measurement of antioxidant. Slurry the alumina with equal parts by volume of chloroform. To prepare a 180 mm × 13 mm i.d. alumina column, place just sufficient glass wool in the bottom of the column to support the column packing, fill the column, and place a small pad of glass wool on top of the column to prevent disturbing the column packing when adding the sample. Connect a liter reservoir to the column in such a manner as to provide a 300 mm head of eluant.

Wash the column with 125 ml of chloroform. Add 20 ml of the chloroform extract to the column. Discard the first 10 ml of effluent after beginning addition of the sample to the column.

Trap the next 50 ml of effluent in a 50 ml volumetric flask. The flow rate was found to average approximately 4.6 ml/min. This fraction contains the BHT. Replace the chloroform eluant with 10 % water in methanol eluant. Do not let the top of the column go dry. Start the 10 % water in methanol as the last of the chloroform goes on the column. Trap the Santonox R or Santowhite Powder in a 100 ml volumetric flask. An average flow rate of 2.8 ml/min was found in trapping the second component in a 100 ml volume.

Determine the absorbance at 283 mμ of the BHT fraction *versus* a chloroform blank. Determine the absorbance of the Santonox R fraction at 280 mμ versus a 10 % water in methanol blank. Determine the absorbance at 282 mμ if the Santowhite Powder is present. In order to correct for small absorbances due to polyethylene species, a sample of polyethylene which contains no additives is carried through the procedure in a similar manner.

3. Calculations. The following equation is used to calculate the per cent antioxidant:

$$\% \text{ Antioxidant} = \frac{(A)\,(V_1)\,(V_2)}{a_s\,(V_3)\,(10)\,(\text{g of sample})}$$

where A is the corrected absorbance at the cited wave length, a_s is the specific absorptivity (BHT, 283 mμ, 9.75; Santowhite Powder, 282 mμ, 12.6; Santonox R, 280 mμ, 19.7 l/g-cm), V_1 is the volume of effluent, V_2 is the volume of chloroform extract, and V_3 is the volume of chloroform extract placed on the column. Owing to inherent differences in instruments, a_s values should be determined in each laboratory.

DISCUSSION AND RESULTS

The specific absorptivities were calculated from the slope of linear absorbance *versus* concentration plots for BHT in chloroform, Santowhite Powder, and Santonox R in 10 % water in methanol. The ultraviolet spectra of the antioxidants are depicted in Fig. 1.

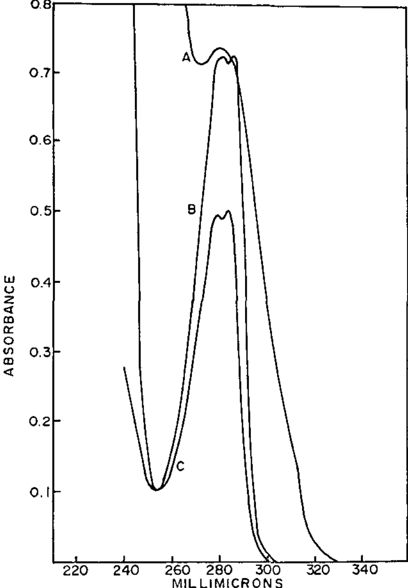

Fig. 1. The U.V. spectra of some antioxidants. A. Santonox R (0.0368 g/l in methanol). B. Santo white Powder (0.0585 g/l in methanol). C. BHT (0.052 g/l in chloroform).

Typical elution chromatograms which show the separation of mixtures of BHT-Santonox R and BHT-Santowhite Powder are depicted in Fig. 2 and Fig. 3, respectively. It is noted that the water-methanol effluent front elutes the bulk of the Santonox R or Santowhite Powder. A large error would result if some of the first part of the

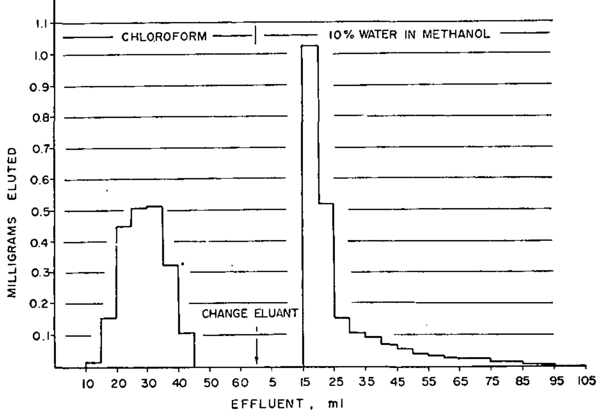

Fig. 2. Elution chromatogram of BHT and Santonox R.

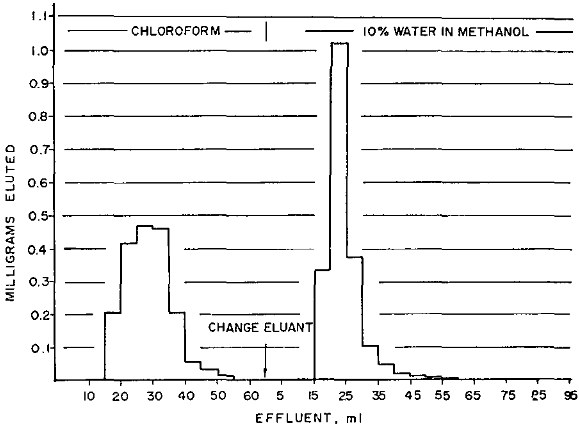

Fig. 3. Elution chromatogram of BHT and Santowhite Powder.

aqueous methanol fraction were discarded. Owing to slight differences in the activity of alumina, it is recommended that the effluent volumes required for separations be determined for each new batch of alumina. An ultraviolet spectrophotometric monitor was found to be a convenient tool for establishing these volumes.

The procedure was verified by analyzing polyethylene samples containing known amounts of antioxidants. These standards were prepared by adding aliquots of known concentrations of antioxidants in ethyl ether to weighed amounts of powdered polyethylene. The ether was allowed to evaporate at room temperature. The mixture was stirred and then pressed for 5 min between two alumina plates at a pressure of 600 p.s.i. at 135°. The analytical results are cited in Table I.

TABLE I

ANALYSIS OF SOME STANDARD POLYETHYLENE SAMPLES

BHT			*Santowhite Powder*			*Santonox R*		
% added	*% found*	*% recovered*	*% added*	*% found*	*% recovered*	*% added*	*% found*	*% recovered*
0.270	0.265	98.0	0.260	0.264	101.3	—	—	—
0.263	0.260	98.8	—	—	—	0.257	0.248	96.5
0.0513	0.0508	99.2	0.253	0.249	98.4	—	—	—
0.0535	0.0534	99.8	—	—	—	0.251	0.245	97.5

Some standard samples were prepared by milling weighed amounts of BHT, Santowhite Powder, and Santonox R in polyethylene at 130°. The analyses of these samples agreed satisfactorily for Santowhite Powder and Santonox R but the BHT concentrations were approximately 30% low. A feasible explanation would be that BHT has a greater volatility than the others, and some BHT was lost due to evaporation during the hot milling process.

Chloroform was chosen as the solvent for extracting the antioxidants from polyethylene because chloroform readily permeates polyethylene and, in addition, it exhibits a favorable distribution coeffcient for phenolic antioxidants[4]. Furthermore, chloroform had the favorable solvent characteristics required of the first eluant in

the adsorption chromatographic separation. The affinity of chloroform for these antioxidants was demonstrated by the fact that essentially none of the antioxidants were extracted from chloroform by 0.1 *N* aqueous sodium hydroxide.

Since short extraction times were desirable, it was found that 3 h of chloroform extraction of the polyethylene samples at 50° gave the results cited in Table I while the same extraction carried out at ambient temperature gave slightly lower recovery.

Chloroform elutes BHT from an alumina column rapidly, but elutes Santonox R and Santowhite Powder slowly enough to permit their complete separation. However, an excessive amount of chloroform (400 ml) was required to completely elute Santowhite Powder and Santonox R. In order to elute these components in a 100 ml volume, it was necessary to change to a more polar solvent system. Methanol eluted Santowhite Powder quite satisfactorily in 100 ml volume, but Santonox R continued to tail. This problem was resolved by using 10% water in methanol. If desired, additional sensitivity may be obtained by concentrating the fractions under vacuum or using greater path length absorption cells.

In the preparation of certain types of polyethylene, it is necessary to add cross-linking agents such as dicumyl peroxide. Dicumyl peroxide elutes in the first chromatographic fraction and can be determined by U.V. absorption measurements. The method would be applicable to the analyses of mixtures of Santowhite Powder with dicumyl peroxide and Santonox R with dicumyl peroxide, but would not apply to a mixture of BHT with dicumyl peroxide. It should be possible by a slight modification of the method to analyze a mixture containing all three antioxidants. Since Santonox R gives a second absorption maximum at 248 mμ (a_s 45.4) and Santowhite Powder has a minimum at 253 mμ, a two component spectrophotometric system could be used to determine Santonox R and Santowhite Powder in the aqueous methanol fraction. Although the work discussed here was done with polyethylene it seems reasonable that the method would be applicable to other polyolefins.

ACKNOWLEDGEMENT

The careful experimental work performed by C. K. HARMON and the consultation provided by A. Y. CORAN in the preparation of polyethylene standards are appreciated.

SUMMARY

An analytical method was developed for the determination of some mixed phenolic antioxidants in polyethylene. It is applicable to the analyses of mixtures of 4,4'-butylidene-bis-(6-*tert.*-butyl-*m*-cresol) with 2,6-di-*tert.*-butyl-*p*-cresol (BHT) and 4,4'-thio-bis-(6-*tert.*-butyl-*m*-cresol) with BHT in polyethylene in the range of 0.01% to 0.3%. The method is based on the separation of the mixed antioxidants by adsorption chromatography with subsequent spectrophotometric determination of the separated antioxidants.

REFERENCES

1 C. W. WADELIN, *Anal. Chem.*, 28 (1956) 1530.
2 C. L. HILTON, *Anal. Chem.*, 32 (1960) 383.
3 C. STAFFORD, *Anal. Chem.*, 34 (1962) 795.
4 H. L. SPELL AND R. D. EDDY, *Anal. Chem.*, 32 (1960) 1811.

SEPARATION AND ESTIMATION OF THE 2,4-DINITROPHENYL DERIVATIVES OF GLUTAMIC AND ASPARTIC ACIDS

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INTRODUCTION

The separation of the 2,4-dinitrophenyl (DNP) derivatives of the naturally occurring amino acids has been reviewed by FRAENKEL-CONRAT, HARRIS AND LEVY[1], BISERTE, HOLLEMAN, HOLLEMAN-DEHOVE AND SAUTIÈRE[2] and by MONIER[3]. In the course of the studies of the incorporation of the radioactive amino acids into proteins a simple method was required for the quantitative separation of the DNP-glutamic and DNP-aspartic acids at the 0.5 to 5 μmole level. Column chromatographic methods of various kinds have been used for the separation of DNP-amino acids. SANGER[4] introduced chromatography on silica gel. BLACKBURN[5] used buffered columns of the same type. MILLS[6] has used specially treated Celite with chloroform–methyl ethyl ketone mixtures. PERRONE[7] introduced ether and chloroform–butanol solvents with buffered columns of Celite 545. BELL *et al.*[8] mention the use of buffered columns of Hyflo Super Cel with ethyl acetate as solvent. MATHESON[9–11] has shown ethyl acetate to be an extremely useful solvent for the chromatography of the DNP-amino acids on buffered columns and had when this work was begun obtained separations of most of the commonly occurring amino acids with the exceptions of DNP-aspartic and DNP-glutamic acids and DNP-glycine and DNP-proline. More recently he has extended his methods to include the separation of these amino acids[10]. WOOLLEY[12] lists some results of the counter current distribution of some DNP-amino acids between ethyl acetate and acetate buffers and ethyl acetate and phosphate buffer. KHOKHLOV AND CH'IH[13,14] have used butyl acetate–phosphate buffer with counter current distribution to separate and estimate several amino acids.

Of the methods described only the method of PERRONE[7] seemed rapid enough for routine use in isolating DNP-glutamic and DNP-aspartic acids. Some difficulty was experienced in packing the columns using ether as a solvent and complete separation of DNP-aspartic acid, DNP-glutamic acid and DNP-serine on a single column was difficult. CALLOW AND WORK[15] experienced similar difficulties in separation with PERRONE'S system and used modified systems for each of the amino acids in which they were interested.

The method to be described is essentially similar to those described by MATHESON[9–11]. The method gives a clear separation between DNP-glutamic and DNP-aspartic acids and removes the derivatives of the other commonly occurring amino acids in a single buffered column of Hyflo Super Cel with a mobile phase of ethyl

acetate–isoamyl alcohol. The method is readily adapted to the estimation of the free amino acids in protein hydrolysates and conditions of quantitative dinitrophenylation are described.

DNP-glutamic and DNP-aspartic acids generally run on paper with very similar R_F values in a variety of solvents[2,3] but can be resolved using phosphate buffer at pH 6 (LEVY[16]) or isoamyl alcohol with acetic acid (BISERTE AND OSTEUX[17]). Conditions are described for the use on paper of the solvent system devised for column chromatography as a useful supplement to the methods of LEVY and BISERTE AND OSTEUX for identification when DNP-hydroxyproline or DNP-*meso*-diaminopimelic acid are present.

METHODS

Column chromatography

Hyflo Super Cel (Johns-Manville and Company) was passed through a sieve of 100 mesh/in., treated with 3 N hydrochloric acid for several hours and washed successively with water and ethanol before drying for 24 h at 120–140°.

The solvent mixture was ethyl acetate–isoamyl alcohol–buffer (10:2:5, by vol.). The buffer was 0.1 M sodium acetate of pH 4.8 to which was added 3 % (w/v) of sodium chloride. All operations were carried out in a room thermostatically controlled at 20° ± 1° in subdued lighting. 4.0 g of the treated Hyflo Super Cel was suspended in 50 ml of the equilibrated organic phase and shaken for 5 min before the gradual addition of 2.5 ml of the aqueous phase and a further period of shaking for 10 min. The columns of 1 cm internal diameter were packed using a stainless steel rod with a perforated disc[18]. The mixture of DNP-amino acid (10–20 µmoles total, up to 3 µmoles each of DNP-glutamic and DNP-aspartic acids) was applied to the column in 0.6 ml of organic phase. The flow of eluant was 0.4/0.5 ml/min.

Dinitrophenyl derivatives of alanine, cysteine, cystine, glycine, leucine, isoleucine, lysine, hydroxylysine, ornithine, phenylalanine, tryptophan and tyrosine were rapidly eluted followed by those of threonine, serine and hydroxyproline with R values of 1.0, 0.9 and 0.7 respectively. Chromatography of DNP-derivatives of *meso*- and LL-diaminopimelic acid gave R values of 0.7 and 1.2 respectively. DNP-glutamic and DNP-aspartic acids were last to be eluted with R values of 0.50 and 0.28 respectively.

The bands of DNP-glutamic and DNP-aspartic acids were completely separated and could be collected as single fractions. The solvent was removed under reduced pressure at 40° and the DNP-glutamic and DNP-aspartic acids dissolved in 0.01 M sodium phosphate buffer of pH 11 for a spectrophotometric estimation at 360 mµ using the molecular extinction coefficients given by LEVY[16].

Preparation of the DNP-derivatives from a mixture of the free amino acids

Reaction of the dicarboxylic amino acids with 1-fluoro-2,4-dinitrobenzene (FDNB) is slow in the presence of other amino acids (*cf.* MILLS[6]) but the following method gave satisfactory recoveries. To the mixture of amino acids (about 15 µmoles total) in 1.0 ml of water were added successively 1.5 ml of ethanol containing 150 µmoles of FDNB and 0.2 ml of 10 % (w/v) sodium bicarbonate; the solution being gently shaken on a water bath at 40° for 6 h. After 4 h a further addition of 0.1 ml of 10 % (w/v) sodium bicarbonate was made. The ethanol was removed under reduced pressure and the excess FDNB and some of the 2,4-dinitrophenol formed were extracted in ether

(3 × 4 ml) and rejected. The DNP-amino acids were extracted in ether (4 × 3 ml) after acidification with 0.5 ml of 5 N hydrochloric acid. The ether was removed by evaporation before chromatography. If an excessive amount of 2,4-dinitrophenol had been formed it was readily removed by adding a little water to the sample in a 50 ml beaker and leaving in a freeze-drying apparatus for 24 h.

Paper chromatography

Sheets of Whatman No. 1 paper (36 × 11 cm) were wetted with the buffer solution by descending chromatography and dried at room temperature (20°). The samples were applied in acetone and the papers equilibrated overnight with the aqueous phase before descending chromatography in the organic phase. The R_F values were somewhat dependent on the degree of dryness of the paper, so reference compounds were run. A chromatogram is shown in Fig. 1.

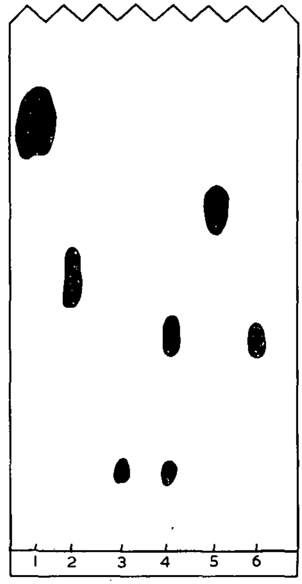

Fig. 1. Chromatogram of DNP-amino acids on Whatman No. 1 paper treated with 0.1 M sodium acetate, pH 4.8 and dried. Mobile phase: ethyl acetate–isoamyl alcohol equilibrated with sodium acetate buffer (10:2:5). (1) DNP-hydroxyproline; (2) DNP-*meso*-diaminopimelic acid; (3) DNP-aspartic acid; (4) DNP-glutamic and DNP-aspartic acids; (5) DNP-serine; (6) DNP-glutamic acid.

RESULTS

Efficiency of separation and recovery

The elution pattern of the DNP-glutamic and DNP-aspartic acids from the buffered columns was determined by collecting 0.8 ml fractions of the eluate. The results are shown in Fig. 2. A series of four experiments were carried out in which known amounts of glutamic and aspartic acids were treated with FDNB and their derivatives chromatographed on the buffered columns with spectrophotometric estimation of the eluate. Recoveries were 100 ± 1.0 S.E.M. and 97 ± 0.7 S.E.M. for aspartic and glutamic acids respectively.

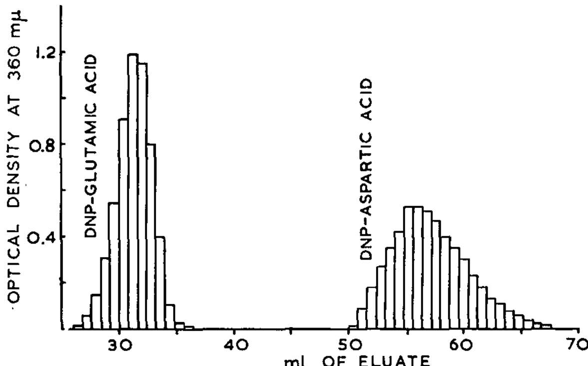

Fig. 2. Plot of absorption at 360 mμ *versus* volume of effluent for a chromatogram of DNP-aspartic and DNP-glutamic acids.

The effect of changes of the pH of the buffer

A series of 0.1 *M* sodium acetate buffers of range 4.4–5.4 were used with the columns to find the optimum pH for the standard method (Table I). Effective separation was obtained at all pH values used with an optimum combination of rapidity of elution with degree of separation at pH 4.8 (before equilibration). The change in *R* value over 1 pH unit as theoretically expected was close to 10 times.

As acetic acid is appreciably soluble in the organic phase the pH of the aqueous phase is considerably altered after equilibration. The change in pH of the aqueous dhase is given by:

$$\text{pH} = \log (1 + \alpha\, V/V^l)$$

where V is the volume of organic phase, V^l the volume of the aqueous phase and α the partition coefficient of the acetic acid in favour of the organic phase. The buffer acts effectively if its p*K* were raised 0.5 units (Table I). Citrate, phosphate and phthalate buffers were used in the same effective range as the acetate. With phosphate and phthalate buffers of the same molarity there was evidence of tailing probably due

TABLE I

RELATIONSHIP OF THE *R* VALUES OF DNP-GLUTAMIC AND DNP-ASPARTIC ACIDS WITH THE pH OF THE STATIONARY PHASE

Column: Hyflo Super Cel; solvent system: ethyl acetate–isoamyl alcohol–0.1 *M* sodium acetate (10:2:5, by vol.).

Original pH of buffer	*pH of buffer after addition of 3% (w/v) sodium chloride*	*pH of stationary phase after equilibration*	*R values*	
			DNP-glutamic acid	*DNP-aspartic acid*
4.4	4.24	4.98	0.75	0.50
4.6	4.44	5.18	0.63	0.39
4.8	4.65	5.39	0.51	0.28
5.0	4.83	5.57	0.25	0.17
5.2	5.04	5.78	0.12	0.07
5.4	5.24	5.98	0.08	0.04

to inadequate buffering capacity as it could be eliminated by increasing the concentration of the buffers. 1.5 *M* phosphate buffers at pH 6 gave very good separations with narrow bands. At the lower range of pH studied (5.4 after equilibration) the resolution and band sharpness with acetate buffer was better than with the other buffers. Acetate buffer was therefore chosen for the routine method as being slightly more flexible.

The addition of isoamyl alcohol gave a slight increase in the separation achieved. The presence of sodium chloride in the buffer facilitated the separation of the two phases after mixing and appeared to give slightly narrower bands on chromatography. Its use in the method is not strictly essential. A sharpening of the bands was observed on increasing the concentration of the acetate buffer to 1 *M*; the standard deviation of the distribution of the solute in the effluent decreased from 1.6 ml to 1.3 ml for DNP-glutamic and from 2.9 ml to 2.3 ml for DNP-aspartic acid without appreciable effect on the *R* values. Increasing the buffer to 2 *M* gave no further improvement in the distribution of DNP-glutamic acid and spread the DNP-aspartic acid to a S.D. of 3.3.

To avoid the difficulty of removing large quantities of acetic acid in the effluent before the spectrophotometric assay in 0.01 *M* sodium phosphate we used 0.1 *M* sodium acetate to buffer the columns in the standard method as the resolution was adequate.

DISCUSSION

The methods described are simple and inexpensive to carry out and should be useful in problems concerning the dicarboxylic amino acids. In our hands the column chromatography was more convenient and gave clearer separations than the method of PERRONE[7].

In contrast to the observations of MATHESON[9,10] in the region of pH 8 the change of *R* value with pH in the region of pH 5 is close to that expected from simple partition of the non-ionised form of the DNP-amino acids.

The superiority of acetate over other buffers at the lower range of pH studied when the *R* values and the concentration of DNP-amino acids in the mobile phase are greater may indicate that there is some association of the DNP-amino acids in the mobile phase which is repressed by the free acetic acid.

ACKNOWLEDGEMENTS

We are indebted to Dr. N. A. MATHESON who generously made available the details of his method for DNP-valine and the chromatographic sequence of the DNP-amino acids on his ethyl acetate columns at pH 8.3 and pH 12 before publication.

We also thank Dr. ELIZABETH WORK who kindly provided the samples of *meso*- and LL-diaminopimelic acids.

One of us (A.V.P.) held a Gulbenkian Foundation Fellowship while this work was being carried out

SUMMARY

DNP-aspartic and DNP-glutamic acids can be separated in the presence of other DNP-amino acids on columns of Hyflo Super Cel with 0 1 *M* sodium acetate (pH 5.4

after equilibration) using ethyl acetate–isoamyl alcohol. Some of the factors influencing the separation have been examined. The solvent system may be adapted to paper chromatography.

REFERENCES

1 H. FRAENKEL-CONRAT, J. I. HARRIS AND A. L. LEVY, *Methods Biochem. Anal.*, 2, (1955) 359.
2 G. BISERTE, J. W. HOLLEMAN, J. HOLLEMAN-DEHOVE AND P. SAUTIÈRE, *J. Chromatog.*, 2 (1959) 225.
3 R. MONIER, in E. LEDERER (Editor), *Chromatographie en chimie organique et biologique*, Vol. II, Masson, Paris, 1960.
4 F. SANGER, *Biochem. J.*, 39 (1945) 507.
5 S. BLACKBURN, *Biochem. J.*, 45 (1949) 579.
6 G. L. MILLS, *Biochem. J.*, 50 (1952) 707.
7 J. C. PERRONE, *Nature*, 167 (1951) 513.
8 P. H. BELL, F. J. BONE, J. P. ENGLISH, C. E. FELLOWS, K. S. HOWARD, M. M. ROGERS, R. G. SHEPARD, R. WINTERBOTTOM, A. C. DORNBUSH, S. KUSHNER AND Y. SUBBAROW, *Ann. N.Y. Acad. Sci.*, 51 (1949) 897.
9 N. A. MATHESON, *Biochem. J.*, 88 (1962) 99P.
10 N. A. MATHESON, *Biochem. J.*, 88 (1963) 146.
11 N. A. MATHESON, *Biochem. J.*, 88 (1963) 151.
12 D. W. WOOLLEY, *J. Biol. Chem.*, 179 (1949) 593.
13 A. S. KHOKHLOV AND CH'IH CH'ANG-CH'ING, *Biokhimiya*, 25 (1960) 1030.
14 A. S. KHOKHLOV AND CH'IH CH'ANG-CH'ING, *Biokhimiya*, 26 (1961) 296.
15 R. K. CALLOW AND T. S. WORK, *Biochem. J.*, 51 (1952) 558.
16 A. L. LEVY, *Nature*, 174 (1954) 126.
17 G. BISERTE AND R. OSTEUX, *Bull. Soc. Chim. Biol.*, 33 (1951) 50.
18 A. J. P. MARTIN, *Biochem. Soc. Symp. (Cambridge, Engl.)*, No. 3 (1949) 4.

CHROMATOGRAPHY OF ORGANIC COMPOUNDS

I. THIN-LAYER CHROMATOGRAPHY OF OLEFINS*

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In recent years, thin layer chromatography[1-3] has acquired an important place in organic laboratory practice, especially due to its versatility and the speed with which separations can be effected. Though various attempts[4-7] at thin-layer chromatography of olefins have been made, results have not been encouraging with olefinic hydrocarbons, due to their rapid movement even with a non-polar solvent like *n*-hexane. In this communication we describe the use of silica gel containing silver nitrate for the thin-layer chromatography of olefinic hydrocarbons, a procedure which we have used with considerable success for the past year or so. Though the results reported in this paper only cover the sesquiterpene hydrocarbons, the method has been used with equal success with other unsaturated hydrocarbons and olefins with functional groups.

The capacity of olefins to complex selectively with the silver cation[8-11] has been exploited for the separation of olefins in column[12], paper[13] and gas-liquid chromatography[14,15] and the present work constitutes its extension to thin-layer chromatography**.

During the exploratory phase of this problem, it was found that a mixture of β-selinene (I) and caryophyllene (II) could be separated satisfactorily on silica gel

(I) (II)

containing 25 % silver nitrate with a solvent system consisting of benzene and 5 % acetone. This system was employed to investigate and standardise the various variables.

Even after taking the usual precautions[17] for ensuring reproducibility in R_F values, the results were not satisfactory and it was soon found out that the mode of preparation of the silver nitrate–silica gel mixture was also an important factor determining the reproducibility of R_F values. A method, as described under procedure, was evolved which always yielded a silver nitrate–silica gel mixture of uniform

* Communication No. 671—National Chemical Laboratory, Poona 8, India.

** Recently BARRETT, DALLAS AND PADLEY[16] have described the use of silica impregnated with silver nitrate for the thin-layer chromatography of glycerides.

quality. Furthermore, it was found that it was better to record the data in terms of movement of a standard substance[18,19] rather than that of the solvent front; two dyes, Sudan III (*p*-phenylazoaniline → 2-naphthol) and azo-benzene were found useful for this purpose and the data are given in terms of R_S or R_A values (R_{Dye} values) depending on whether the reference substance is Sudan III (S) or azo-benzene (A) respectively. R_{Dye} is defined by the expression:

$$R_{Dey}\ (R_S \text{ or } R_A) = \frac{\text{Movement of substance from start in mm}}{\text{Movement of dye from start in mm}}$$

In all experiments the solvent was allowed to rise to a height of 10 cm.

EXPERIMENTAL

Effect of mode of activation on R_S values

The absolute value of R_{Dye} (for a given compound) and the degree of deviation in a set of experiments was found to be dependent on the mode of activation of the plates. Table I summarises the data. On the basis of these results, the method of activation as used in the last set of experiments was adopted for subsequent work. Plates activated in this way could be stored in a desiccator in the dark for two to three weeks without affecting their performance.

TABLE I

EFFECT OF MODE OF ACTIVATION ON R_S VALUES

Mode of activation* (after initial drying in air for 3 h)	*Caryophyllene*			*β-Selinene*		
	Number of experiments	Mean R_S value	Maximum deviation	Number of experiments	Mean R_S value	Maximum deviation
1/2 h at 105°; cool 1 h	4	0.289	+0.021 —0.007	4	0.698	+0.022 —0.007
2 h at 105°; cool 12 h	24	0.282	+0.029 —0.c66	24	0.601	±0.027
2 1/2 h at 105°; cool 1 h	4	0.257	+0.027 —0.016	4	0.587	+0.040 —0.020
2 1/2 h at 105°; cool 12 h	15	0.259	+0.011 —0.012	15	0.586	+0.019 —0.015

* Cooling was carried out in a desiccator (silica gel).

Silver nitrate content and R_S values

In order to determine the dependence of the R_S values on the silver nitrate content of the silica gel, a series of mixtures of silica gel containing different amounts of silver nitrate was prepared and the R_S values for caryophyllene and β-selinene (in a mixture) determined. Fig. 1 depicts the data graphically. As anticipated the R_S value is dependent on the silver nitrate content, but the ΔR_S for the two compounds does remain more or less constant. For subsequent work silica gel containing 15 % silver nitrate was selected as it offered a better distribution of the spots for a solvent rise of 10 cm.

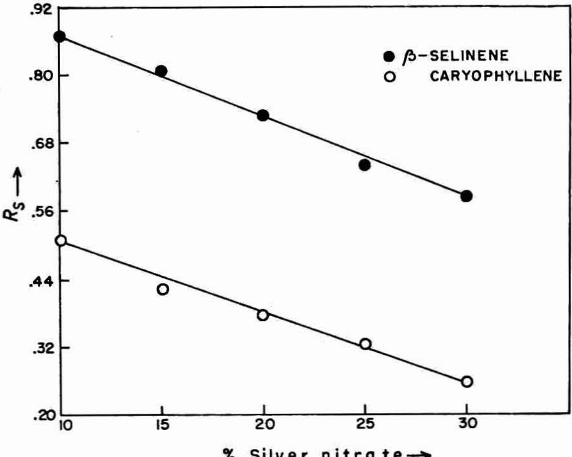


Fig. 1. Variation of R_S with the silver nitrate content.

Solvent system

Having standardised the mode of activation of the plates and the method of preparation of silica gel–silver nitrate mixture, attention was given to the determination of R_S values in different solvents so as to arrive at the most suitable solvent system. Based on the results in Table II benzene containing 5 % acetone was selected as the

TABLE II

APPROXIMATE R_S VALUES OF CARYOPHYLLENE AND β-SELINENE IN DIFFERENT SOLVENTS

(at 28° ± 2°)

Solvent	*Caryophyllene*	*β-Selinene*
Benzene	0.285	0.807
Toluene	0.299	0.966
Acetone	0.712	0.896
Ethyl acetate	0.609	0.848
Dioxane	0.249	0.623
Di-isopropyl ether	0.764	1.065
Hexane	No movement of dye and compounds	
CH_2Cl_2	No movement of compounds	
$CHCl_3$	Very slight movement of compounds	
CCl_4	Very slight movement of compounds	
Benzene + 5 % acetone	0.422	0.805
Toluene + 5 % acetone	0.470	0.844

most suitable solvent mixture for determining the R_S values of some other sesquiterpenes.

R_{Dye} values for some sesquiterpenes

The thin-layer chromatography of olefins, under the conditions described above, was next applied to a larger number of sesquiterpenes, when it was found that certain sesquiterpenes moved along with the solvent front (benzene + 5 % acetone). How-

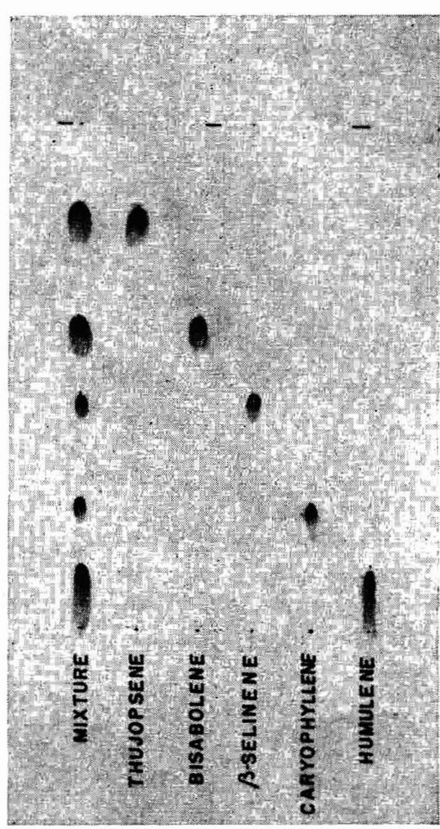


Fig. 2. Thin-layer chromatogram of some sesquiterpenes.

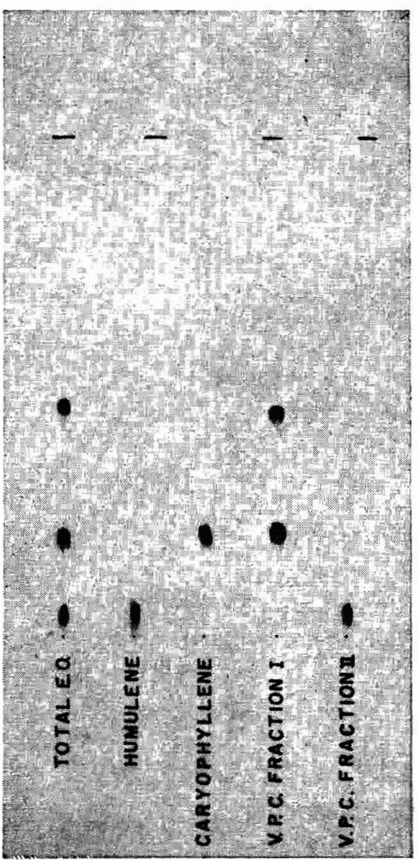


Fig. 3. Thin-layer chromatogram of the essential oil of *Dipterocarpus pilosus* and its fractions.

ever, these compounds could be separated satisfactorily by using *n*-hexane as the developing solvent; since Sudan III does not move at all with the solvent, azobenzene was used as the reference dye and as stated earlier these data are recorded as R_A values. The R_S and R_A values of some sesquiterpenes are given in Table III and Fig. 2 shows a typical thin-layer chromatogram of a mixture of sesquiterpenes.

Thin layer chromatography of sesquiterpenes on silica gel–silver nitrate, in conjunction with gas–liquid chromatography (GLC), has proved of value in the study of essential oils, both in identifying the various components and also in indicating whether a single peak in a gas–liquid chromatogram represents one component or, possibly, is still a mixture. For example, Fig. 3 shows a thin-layer chromatogram of the essential oil from *Dipterocarpus pilosus*; its GLC over diethylene glycol showed only two peaks, which were perfectly symmetrical, however, thin-layer chromatography showed that the first fraction contained another component, besides caryophyllene. The identification of humulene and caryophyllene in the essential oil was aided by determining the retention times and the R_S values of its components and comparing them with standard values.

TABLE III

R_{Dye} VALUES OF SOME SESQUITERPENES (at 28° ± 2°)

Compound	No. of olefinic linkages in the molecule	R_S*	R_A†
Humulene	3	0.189	—
β-Elemene	3	0.331	—
Caryophyllene	2	0.422	—
β-Selinene	2	0.805	—
β-Bisabolene	3	0.936	—
Thujopsene	1	1.149	—
Copaene	1	1.161	—
ar-Himachalene	one aromatic ring	1.164	—
α-Gurjunene	1	1.168	—
β-Himachalene	2	—	3.155
α-Himachalene	2	—	5.190
Longifolene	1	—	5.357
Cuparene	one aromatic ring	—	5.391
Iso-longifolene	1	—	7.447
Longicyclene	nil	—	8.399

* Solvent system: Benzene + 5 % acetone.
† Solvent system: n-Hexane.

PROCEDURE

Materials

Silica gel used for this work was "Silica gel less than 0.08 mm for chromatography"*. Sieve analysis showed that 97 % passed through 200 mesh.

The terpene hydrocarbons were pure samples, either available in this laboratory or supplied by the courtesy of other workers.

Azobenzene (1 g) was purified by passing its benzene solution through alumina (activity I, 30 g) and further eluting with benzene. Sudan III was first crystallised from ethanol and then purified as above. Purified materials gave single spots in thin-layer chromatography (silica gel/benzene).

Only purified anhydrous solvents were employed.

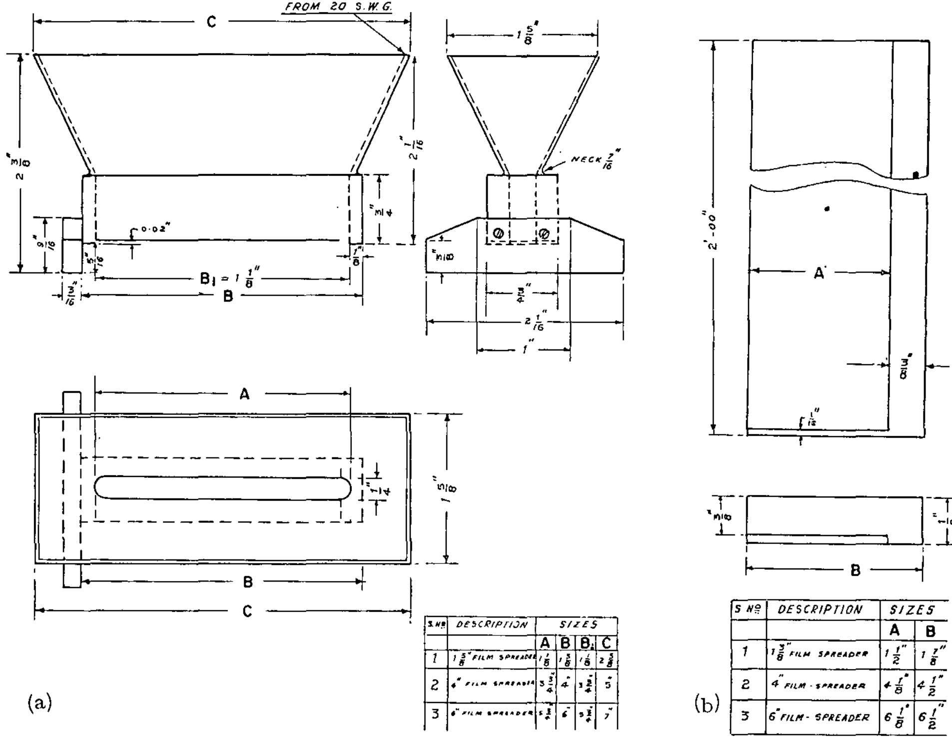
Silica gel–silver nitrate mixture

An aqueous solution of silver nitrate (National Refinery, India) 7.5 g in 7.5 ml water, was diluted with 125 ml alcohol and 50 g silica gel was added gradually to this, with stirring. After stirring for a further 15 min, the aqueous alcohol was removed by suction (water-bath) with constant shaking and the residue finally dried in vacuum to constant weight. Plaster of paris (—200 mesh) was added to this dry powder, 7.5 g for every 57.5 g of silica gel–silver nitrate, and this was well mixed by thoroughly shaking in a mechanical shaker. The tan-coloured preparation, if stored in the dark, will keep well for a few months.

* E. Merck A.G., Darmstadt, Germany.

General conditions

Smooth glass plates (thickness 0.3 cm) in two sizes (3.5 × 15 cm and 10.0 × 15 cm) were used. The silica gel–silver nitrate mixture (1 part) was slurried with water (2.5 parts) by gently rubbing in a pestle and mortar and the plates coated with the slurry with the aid of an applicator (Fig. 4). Two different sizes of applicators were used for the two sizes of glass plates. The applicators, which can be cheaply and easily constructed, are made in stainless steel. The design is based on "Sheen's film applicator for paints and varnishes"*. The applicators give a uniform film of 0.5 mm and 0.3 mm thickness with the smaller and the large applicator respectively, and the hopper dimensions are such that with a slurry made from 4.0 g or 6.0 g of the material, four smaller or four larger plates can be made with the appropriate applicator.



S.No.	DESCRIPTION	SIZES			
		A	B	B1	C
1	1 5/8" FILM SPREADE	1 1/8	1 5/8	1 1/8	2 5/8
2	4" FILM SPREADER	3 3/4	4"	3 3/4	5"
3	6" FILM SPREADER	5 3/4	6"	5 3/4	7"

S No.	DESCRIPTION	SIZES	
		A	B
1	1 3/8" FILM SPREADER	1 1/2"	1 7/8"
2	4" FILM-SPREADER	4 1/8"	4 1/2"
3	6" FILM-SPREADER	6 1/8"	6 1/2"

Fig. 4. Stainless steel applicator.

The plates were dried in the air for 3 h and then activated at 105° for 2$^1/_2$ h and finally stored in a desiccator over silica gel until needed.

The starting points were marked on the plates at 2 cm from the lower edge with a distance of 1 cm in between.

1–10 μl of a 1 % solution of the substance in hexane (dyes were applied as 0.2 % solution in acetone) was applied at the starting points. After a few minutes, the plates were placed in glass cylinders (18.5 cm × diam. 5 cm) having a 1 cm high segment of a

* Sheen Instruments Ltd., London.

glass tube (diam. 3 cm) to act as a stand for the plate. The cylinders contained the appropriate solvent to a height just below that of the plate stand. These were tightly closed with a cork with a small plugged hole, through which more solvent could be introduced later. The jars, also, contained a semi-cylindrical strip of filter-paper of height almost equal to that of the cylinder in order to help in the saturation of the cylinder atmosphere with the solvent vapours[18]. The larger plates were placed in a glass jar (9.8 cm × 15.0 cm × 19.8 cm) fitted with a suitable glass stand to hold two such plates, and carrying a filter-paper strip along the sides (height 15 cm) immersed in the solvent, and closed by a ground-glass cover. After the plates had been allowed to stand for 15 min to equilibrate, more solvent was added so as to wet the glass plates to a height of about 0.5 cm. Development was stopped when the solvent reached the pre-marked height of 10 cm; development took about 20–25 min.

After development the plates were dried in air (20 min) and the dried plates were sprayed with a solution of chlorosulphonic acid in acetic acid (1:2)[20] and heated for 10 min at 130°. Usually different coloured spots were observed, *e.g.* humulene, brown; caryophyllene, blue; longifolene, pink.

ACKNOWLEDGEMENTS

We wish to thank Prof. ERDTMAN for the supply of thujopsene and cuparene, Prof. SORM for the β-elemene and Prof. OURISSON for the α-gurjunene.

SUMMARY

A method for the thin-layer chromatography of olefins over a silica gel–silver nitrate mixture is described.

REFERENCES

1 E. DEMOLE, *J. Chromatog.*, 1 (1958) 24, 6 (1961) 1.
2 E. G. WOLLISH, M. SCHMALL AND M. HAWRYLYSHYN, *Anal. Chem.*, 33 (1961) 1138.
3 E. STAHL, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin, 1962.
4 J. G. KIRCHNER, J. M. MILLER AND G. J. KELLER, *Anal. Chem.*, 23 (1951) 420.
5 J. M. MILLER AND J. G. KIRCHNER, *Anal. Chem.*, 25 (1953) 1107.
6 R. H. REITSEMA, *Anal. Chem.*, 26 (1954) 960.
7 L. H. BRYANT, *Nature*, 175 (1955) 556.
8 H. J. LUCAS AND W. F. EBERZ, *J. Am. Chem. Soc.*, 56 (1934) 460.
9 S. WINSTEIN AND H. J. LUCAS, *J. Am. Chem. Soc.*, 60 (1938) 836.
10 W. O. JONES, *J. Chem. Soc.*, (1954) 1808.
11 P. D. GARDNER, R. L. BRANDON AND N. J. NIX, *Chem. Ind. (London)*, (1958) 1363.
12 H. L. GOERING, W. D. CLOSSON AND A. C. OLSON, *J. Am. Chem. Soc.*, 83 (1961) 3507.
13 H. S. BARRETO AND C. ENZELL, *Acta Chem. Scand.*, (15 (1961) 1313.
14 B. W. BRADFORD, D. HARVES AND D. E. CHALKLEY, *J. Inst. Petrol.*, 41 (1955) 80.
15 R. R. SAUERS, *Chem. Ind. (London)*, (1960) 176.
16 C. B. BARRETT, M. S. J. DALLAS AND F. B. PADLEY, *Chem. Ind. (London)*, (1962) 1050.
17 M. BRENNER, A. NIEDERWIESER, G. PATAKI AND A. R. FAHMY, *Experientia*, 18 (1962) 101.
18 J. H. DHONT AND C. DE ROOY, *Analyst*, 86 (1961) 74, 527.
19 B. J. R. NICOLAUS, *J. Chromatog.*, 4 (1960) 384.
20 R. TSCHESCHE, W. FREYTAG AND G. SNATZKE, *Ber.*, 92 (1959) 3053.

THIN-LAYER CHROMATOGRAPHY OF ALIPHATIC 2,4-DINITROPHENYLHYDRAZONES

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Carbonyl compounds play an important role in desirable and undesirable flavours. No comprehensive study of aliphatic 2,4-dinitrophenylhydrazones by thin-layer chromatography (TLC) has been published. This paper describes methods for separating members of homologous series and for separating different homologous series from one another by TLC.

I. SEPARATION OF MEMBERS OF AN HOMOLOGOUS SERIES

In 1952 ONOE[1] separated the 2,4-dinitrophenylhydrazones of *n*-aliphatic aldehydes up to C_{10}, propenal, but-2-enal and citral on silica gel plates using benzene saturated with water as developing solvent. Later DHONT AND DE ROOY[2] used a 3:1 mixture of benzene and light petroleum (b.p. 60–80°) to separate the 2,4-dinitrophenylhydrazones of *n*-aliphatic aldehydes, and benzene containing 5 % ethyl acetate to separate the 2,4-dinitrophenylhydrazones of aromatic aldehydes, both on silica gel plates. ROSMUS AND DEYL[3] seaprated the 2,4-dinitrophenylhydrazones of some aldehydes and ketones on alumina using either benzene–hexane (1:1) or ether as solvent. The present author tried these systems on the homologous series of *n*-alk-1-en-3-ones but could not separate homologues above C_6.

KAUFMANN AND MAKUS[4], working in the lipid field, used adsorption TLC to separate substances into classes, but found that partition TLC with impregnated plates was more suitable for separation of members of homologous series. FORSS AND STARK[5], in their discussion of the separation of 2,4-dinitrophenylhydrazones by four[6–9] paper chromatographic systems consider the phenoxyethanol–light petroleum (b.p. 100–120°) system[6] the most satisfactory. This system was therefore tried on kieselguhr G plates with the result that separations of all the members of the normal homologous series of alkanals C_{1-14}, alkan-2-ones C_{3-13}, alk-1-en-3-ones (vinyl ketones) C_{4-10}, alk-2-enals $C_{3-11,16}$, alka-2,4-dienals $C_{5-12,14,16,18}$ and alk-3-en-2-ones $C_{6,7,10}$ were obtained as shown in Fig. 1. A slight difference in R_F value between the *trans*-2,*trans*-6- and *trans*-2,*cis*-6-isomers of nonadienal was also obtained, as illustrated. However, this pair is readily separated by the system described in Section II (B).

Experimental

Dry kieselguhr G plates* (E. Merck A. G., Darmstadt) were rocked in a shallow dish

* The Desaga (Heidelberg, Germany) equipment was used throughout.

filled with a 10% solution of 2-phenoxyethanol in acetone. The impregnated plates were removed, allowed to dry and ether solutions of the 2,4-dinitrophenylhydrazones were applied 4 cm from one edge of the plate. The plate was developed with light petroleum (b.p. 100-120°, not aromatic-free) in a tank lined with filter paper soaked with the light petroleum. The plate was removed from the tank and, after the light

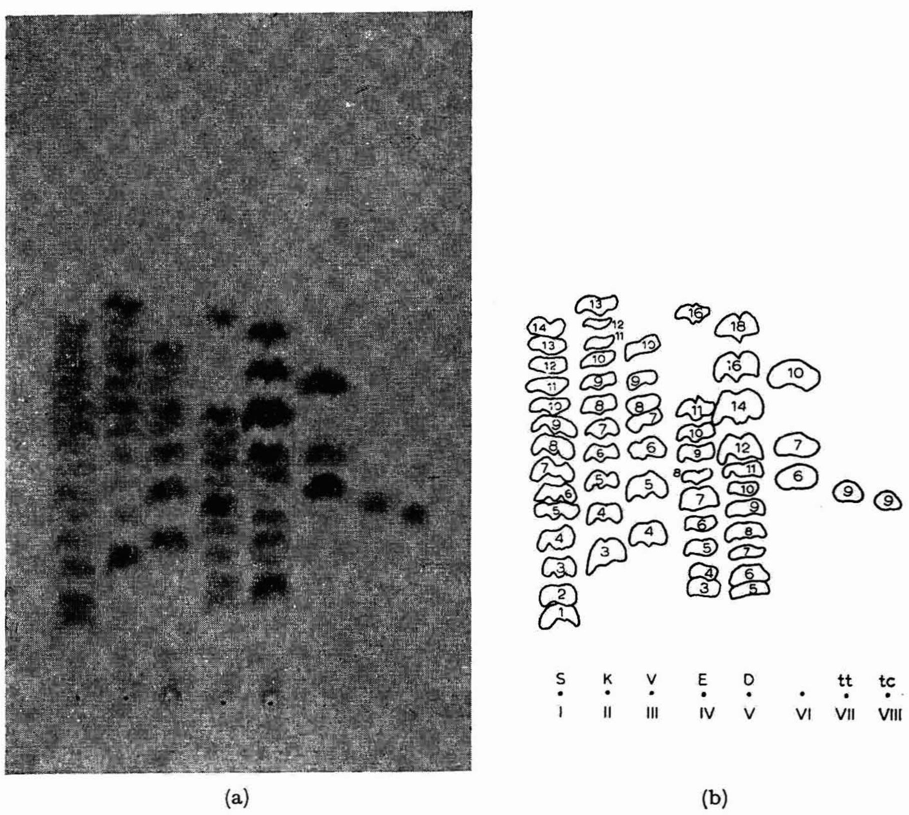


Fig. 1. Separation of members of normal homologous series of 2,4-dinitrophenylhydrazones of (i) alkanals (S) C_{1-14}; (ii) alkan-2-ones (K) C_{3-13}; (iii) alk-1-en-3-ones (V) C_{4-10}; (iv) alk-2-enals (E) $C_{3-11 \cdot 16}$; (v) alka-2,4-dienals (D) $C_{5-12\ 14 \cdot 16 \cdot 18}$; (vi) alk-3-en-2-ones $C_{6 \cdot 7 \cdot 10}$; (vii) nona-*trans*-2,-*trans*-6-dienal (viii) nona-*trans*-2,*cis*-6-dienal. Carrier: kieselguhr G. Impregnation: 10% phenoxyethanol in acetone. Solvent: light petroleum (b.p. 100–120°). Conditions: the mobile solvent was allowed to ascend 9 cm from the starting line three times and 11 cm from the starting line the fourth time.

petroleum had evaporated, was photographed and replaced in the tank. In this way, the solvent was allowed to ascend the plate four times. Figs. 1–4 show the effect of multiple ascent. Obviously, fewer ascents are needed for simpler mixtures. In multiple development, compounds with a high R_F value tend to become crowded together whereas compounds with low R_F values are further separated. This effect has been discussed by LENK[10] who also pointed out that with multiple development, as opposed to longer running distance, the spots become flattened out and hence resolution is improved.

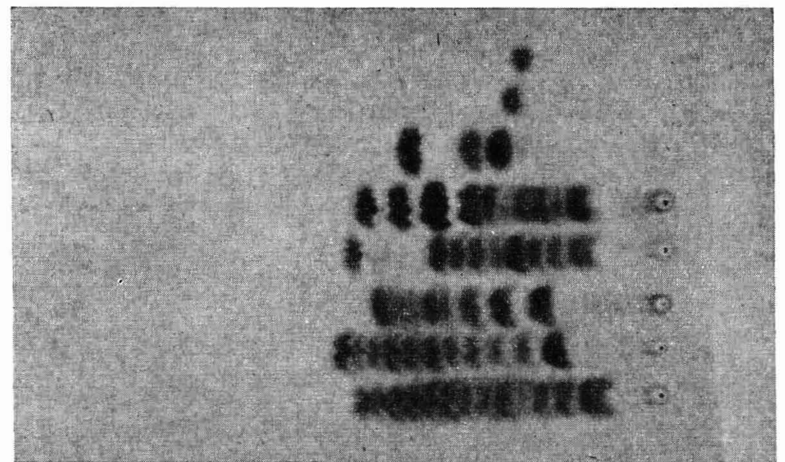


Fig. 4. The plate as in Fig. 1 after three ascents.

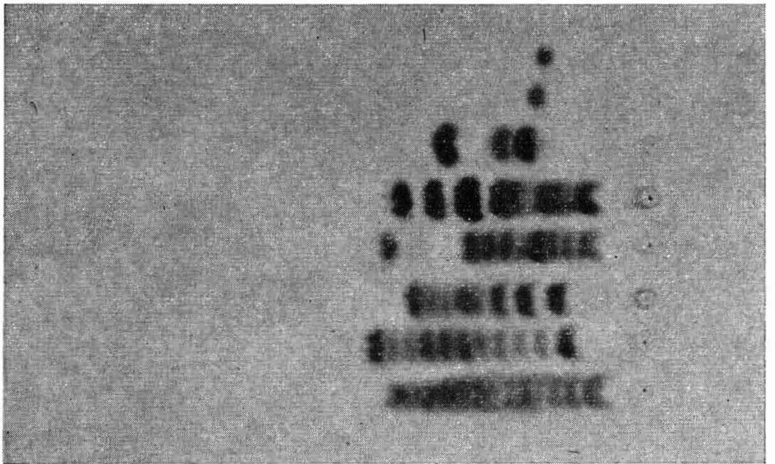


Fig. 3. The plate as in Fig. 1 after two ascents.

Fig. 2. The plate as in Fig. 1 after one ascent.

At the bottom of the plate the phenoxyethanol has been eluted by the light petroleum as shown by the pale area in the photographs, particularly Fig. 1. In order that the eluted area should not interfere with the separation, the starting line was made 4 cm above the edge of the plate instead of the usual 1 cm.

The plates should be photographed within 24 h as the spots tend to diffuse in the phenoxyethanol, and alka-2,4-dienals fade on standing. Orthochromatic film was used for Figs. 1–4 whilst Figs. 5–8 were photographed in U.V. light with a Polaroid camera.

The system described above separates individual members of any homologous series but it is apparent from Fig. 1 that there are several groups of 2,4-dinitrophenylhydrazones from different homologous series which have the same R_F values in this system. The separations of such groups are described in the following sections.

II. SEPARATION INTO CLASSES

(A) *Separation into aldehyde, saturated methyl ketone and vinyl ketone 2,4-dinitrophenylhydrazones*

FORSS, DUNSTONE AND STARK[11] used acid-washed alumina columns to separate 2,4-dinitrophenylhydrazones according to classes. This suggested that some separation into classes should be possible on thin layers of alumina. It was found that 4% diethyl ether in light petroleum readily separated aldehydes, saturated methyl ketones and vinyl ketones but only gave slight separation of aldehydes of different degrees of unsaturation. A separate system, described in Section II (B) was therefore developed for the separation of aldehydes into classes.

Experimental

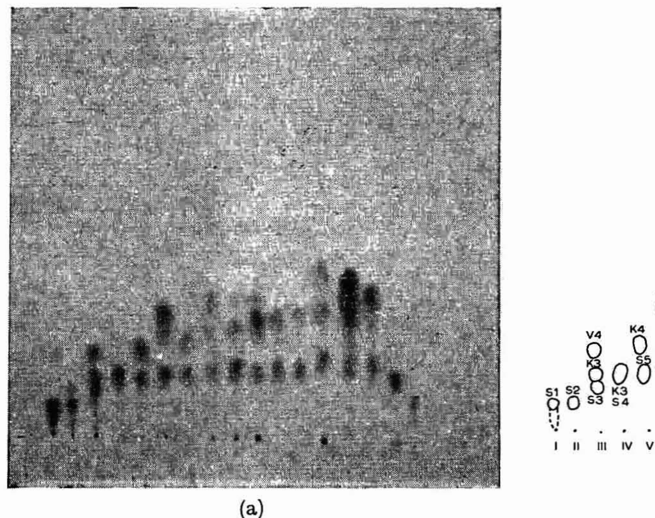
Plates were prepared from a slurry consisting of 30 g of aluminium oxide G (E. Merck A. G., Darmstadt) to 50 ml of distilled water. The plates were allowed to set for 10 min, dried in an oven at 115° for 15 min and stored open to the atmosphere. The duration and temperature of heating of the plates were not found to be critical provided the plates were allowed to equilibrate with the atmosphere. Once the plates were so equilibrated, they could be stored indefinitely, open to the atmosphere.

Compounds were applied in CCl_4 solution. The solvent used was 4% diethyl ether in light petroleum (b.p. 30–40°). The solvent was allowed to ascend to the top of the plate. The tank need not be sealed completely air-tight, in fact, a leak in the seal increases separation. The boiling range of the light petroleum is not critical. Similar separations were obtained with 4% ether in light petroleum (b.p. 100–120°). The spots are intensified and turned bright red with 2% NaOH in ethanol–water (9:1, v/v)[12].

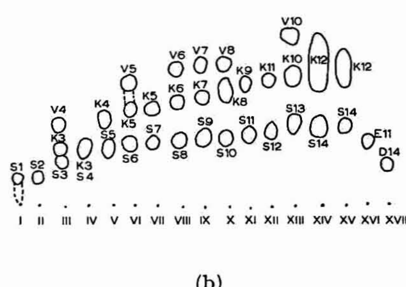
Fig. 5 shows typical separations of groups of *n*-alkanals, *n*-alkan-2-ones and *n*-alk-1-en-3-ones which are inseparable with phenoxyethanol (Section I).

It can be seen that from C_4 onwards all the *n*-alkanals have the same R_F value whereas the R_F's of the *n*-alkan-2-ones increase slightly from C_4 to C_{12} and the R_F's of the *n*-alk-1-en-3-ones increase slightly from C_5 to C_{10}. In both series of ketone derivatives the first member of the series has an appreciably lower R_F value than the second member. Thus the methyl vinyl ketone 2,4-dinitrophenylhydrazone has the

same R_F value as the methyl ethyl ketone derivative and acetone 2,4-dinitrophenylhydrazone has the same R_F value as the alkanal derivatives from butanal onwards and it does not separate very well from the propanal derivative. The pairs not separated on this adsorbent separated well on the adsorbent described in the next section.



(a)



(b)

Fig. 5. Separation of mixtures of normal 2,4-dinitrophenylhydrazones of alkanals (S), alkan-2-ones (K) and alk-1-en-3-ones (V) which have the same R_F value in the partition system illustrated in Fig. 1. (a) Photograph. (b) Tracing. (xiv) dodecan-2-one has been spotted too heavily; (xvi) undec-2-enal; (xvii) tetradeca-2,4-dienal. Adsorbent: aluminium oxide G. Solvent: 4 % diethyl ether in light petroleum (b.p. 30–40°), one ascent to edge of plate.

In general there was poor separation between aldehydes of different degrees of saturation. However, Fig. 5 shows that some separation has been obtained between *n*-tetradecanal, *n*-undec-2-enal and *n*-tetradeca-2,4-dienal.

(*B*) *Separation of aldehydes into alkanals, alk-2-enals, alka-2,4-dienals and nona-2,6-dienals*

Dr. J. H. RUSSEL* drew the attention of the author to a paper by BARRETT, DALLAS AND PADLEY[13] describing the use of silica gel impregnated with silver nitrate to separate isomeric glycerides and glycerides having the same number of carbon atoms but different degrees of unsaturation. Alumina containing 25 % $AgNO_3$ was therefore tried, with success, for the separation of aldehydes into classes. This adsorbent also separated the inseparable pairs of compounds listed in the previous section.

Experimental

Plates were prepared from a slurry made from 30 g aluminium oxide G suspended in a solution of 7.5 g of $AgNO_3$ in 50 ml of water. The plates were allowed to set for 10 min and then heated for 20 min, the oven temperature rising gradually from 115° to 135°. The plates were stored overnight open to the atmosphere, protected from bright light.

* Division of Organic Chemistry, C.S.I.R.O., Melbourne.

Compounds were spotted from CCl_4 solution. The plates were developed with freshly prepared 16% ether in light petroleum (b.p. 30–40°) in tanks lined with filter paper soaked with solvent. Fresh solvent was used for each run as it was found that used solvent gave unsatisfactory separation.

Decreasing the moisture content of Al_2O_3–$AgNO_3$ plates decreases the R_F value and increases the stability of $AgNO_3$ complexes. Thus it would be expected that plates with a low activity would give R_F values in an order similar to that found on an Al_2O_3 plate containing no $AgNO_3$. This was found to be so. On relatively wet plates ketones travel ahead of aldehydes and there is very little separation between the classes of aldehydes, particularly alkanals and alk-2-enals. With decreasing water content the effect of complexing becomes more evident. Apparently alkan-2-one 2,4-dinitrophenylhydrazones form complexes more readily than the alkanal derivatives and therefore the R_F values of alkan-2-one derivatives are decreased to less than those of alkanal derivatives. In both cases complex formation must be assumed to occur at the C=N bond. In this case, the R_F value of the vinyl ketone derivative is also decreased relative to that of the methyl ketone derivative, the additional double bond of the vinyl compound causing the greater decreases in R_F value in the presence of $AgNO_3$.

Several factors influence the stability of the $AgNO_3$ complex.

1. The stability increases with increase in the number of double bonds[14]—the R_F values decrease in the order alkanal, alkenal, alkadienal.

2. Compounds with conjugated double bonds form less stable complexes than compounds with unconjugated double bonds[14]—the nona-2,6-dienals have a lower R_F value than the alka-2,4-dienals.

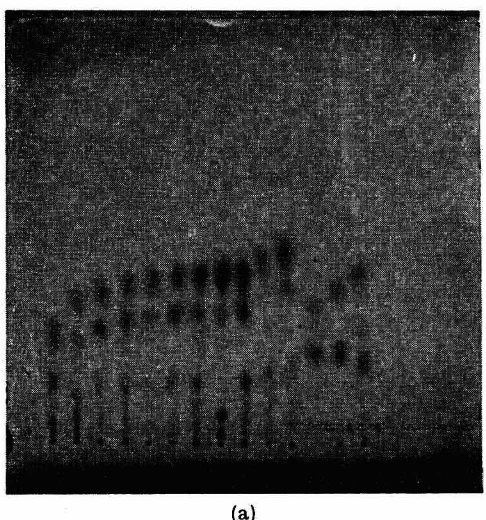
3. *cis*-Double bonds are complexed more readily than *trans*-double bonds[15]—nona-*trans*-2,*trans*-6-dienal has a higher R_F value than nona-*trans*-2,*cis*-6-dienal.

If one bears these factors in mind it will be apparent that if compounds forming relatively stable complexes, such as the *trans,trans* and *trans,cis* forms of nona-2,6-dienal are to be separated, a plate of lower activity is preferable, whereas a wider separation of the relatively less stable complex forming alkanals and alk-2-enals will be obtained with a drier plate. However, all five classes of compounds can be separated on one plate as shown in Fig. 6. There is a wide range of moisture contents which will give a satisfactory separation of alkanals, alk-2-enals and alka-2,4-dienals. As the position of ketones varies from R_F values less than alk-2-enals to R_F values greater than alkanals with small changes in water content it is therefore desirable before an unknown mixture is examined, to remove ketones by using an Al_2O_3 plate.

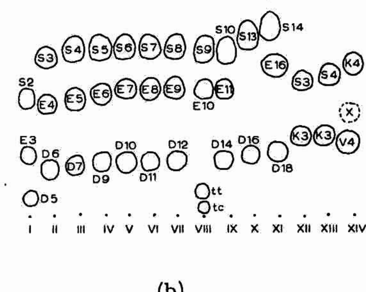
If a group of plates as prepared is found to be too wet, as indicated by poor separation between alkanals and alk-2-enals, a satisfactory plate is often produced by heating for 1 min at about 115°. A plate which has been somewhat overheated will usually give a satisfactory separation with two ascents.

Fig. 6 shows typical separations of groups of aldehyde 2,4-dinitrophenylhydrazones which do not separate on phenoxyethanol-impregnated kieselguhr and only slightly on alumina. The R_F values in each group decrease with increase in the number of double bonds. Except for the very early members of each homologous series R_F values on any one plate are characteristic of a particular series. Thus, providing one member of each series has been added as marker (preferably internal) and ketone derivatives are known to be absent (from a previous chromatogram run on alumina),

aldehyde 2,4-dinitrophenylhydrazones can be classified according to degree of unsaturation, and position and configuration of double bonds. The figure also shows the separation of nona-*trans*-2,*trans*-6-dienal from nona-*trans*-2,*cis*-6-dienal 2,4-dinitrophenylhydrazone, the *trans*-isomer travelling ahead of the *cis*-isomer. The nona-2,6-dienal 2,4-dinitrophenylhydrazones are well separated from the alka-2,4-dienal



(a)



(b)

Fig. 6. Separation of normal 2,4-dinitrophenylhydrazones of alkanals (S); alk-2-enals (E); alka-2,4-dienals (D); (viii) nona-*trans*-2,*trans*-6-dienal (tt) and nona-*trans*-2,*cis*-6-dienal (tc); (xii) propanal (S3) and acetone (K3); (xiii) butanal (S4) and acetone (K3); (xiv) methyl ethyl ketone (K4) and methyl vinyl ketone (V4). (X is an impurity.). Adsorbent: aluminium oxide G containing 20% $AgNO_3$ (w/w). Solvent: 16% diethyl ether in light petroleum (b.p. 30–40°) freshly prepared, allowed to ascend twice to the edge of the plate. (a) Photograph. (b) Schematic drawing.

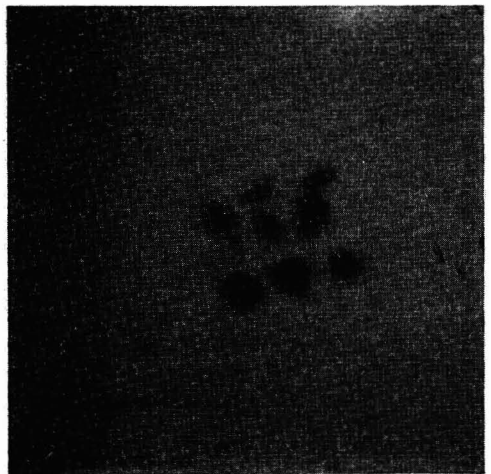
derivatives. The R_F value of acetone 2,4-dinitrophenylhydrazone is always considerably lower than that of the other members of the series. Because of this the Al_2O_3–$AgNO_3$ system can be used to achieve a wide separation between acetone 2,4-dinitrophenylhydrazone and the propanal or butanal derivatives. Fig. 6 illustrates these separations and also that of methyl vinyl ketone and methyl ethyl ketone 2,4-dinitrophenylhydrazones. This pair does separate on the phenoxyethanol system but not on alumina plates. On the Al_2O_3–$AgNO_3$ absorbent a good separation is achieved with one ascent.

A further aid in the identification of classes is the rate at which they blacken. *n*-Alkanal 2,4-dinitrophenylhydrazones became black within a day while 2,4-dinitrophenylhydrazones of unsaturated compounds take several days to blacken. 2,4-Dinitrophenylhydrazones of *n*-alkan-2-ones do not become black at all but eventually fade completely.

III. TWO-DIMENSIONAL APPLICATION

The systems described in the previous sections can be readily combined in a two-dimensional application to separate a mixture containing several members of different homologous series. Two such separations are illustrated in Figs. 7 and 8.

Fig. 7 shows the separation of a mixture containing *n*-alkanal $C_{6,9,13}$ (S6, S9, S13), *n*-alkan-2-one $C_{5,7,10}$ (K5, K7, K10), *n*-alk-1-en-3-one $C_{5,7,10}$ (V5, V7, V10) 2,4-dinitrophenylhydrazones using aluminium oxide G as adsorbent. The plate was developed twice to the edge of the plate in the first direction with 4% diethyl ether in light petroleum (b.p. 30–40°) as solvent. Before development in the second direction the plate was dipped rapidly but steadily and evenly into a 10% solution of 2-phenoxyethanol in acetone. Great care must be exercised in this impregnation to



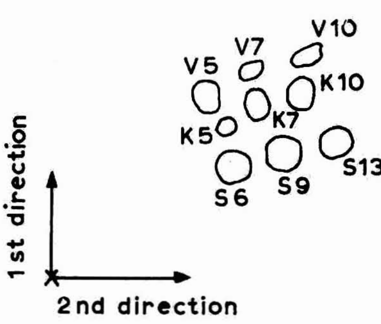
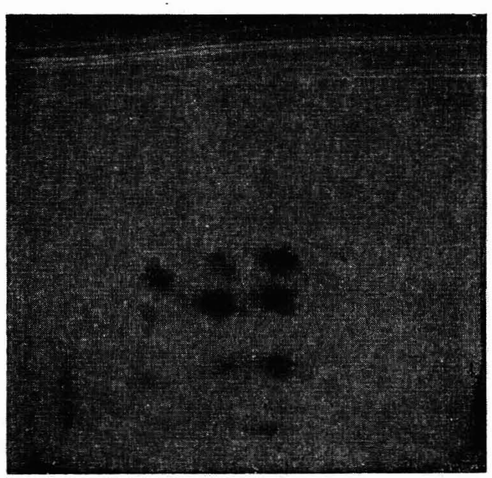


Fig. 7. Two-dimensional separation of normal 2,4-dinitrophenylhydrazones of hexanal (S6); nonanal (S9); tridecanal (S13); pentan-2-one (K5); heptan-2-one (K7); decan-2-one (K10); pent-1-en-3-one (V5); hept-1-en-3-one (V7); dec-1-en-3-one (V10). Adsorbent: aluminium oxide G. Solvent: *first direction* 4% diethyl ether in light petroleum (b.p. 30–40°) allowed to run twice to the edge of the plate; *second direction* plate dipped in 10% phenoxyethanol in acetone and developed once with light petroleum (b.p. 100–120°) to the edge of the plate.

avoid washing off the spots. The plate shoul dbe dipped in the impregnating solution to just above the line of spots and then rapidly withdrawn from the solution. After impregnation of the plate, the acetone was allowed to evaporate off and the plate was developed with light petroleum (b.p. 100-120° not aromatic-free) in a direction at right angles to the first direction. In this case the solvent was allowed to ascend to the edge of the plate. For an unknown mixture, markers can be spotted along the edge of the plate before development in the second direction. The fact that spots appear to streak after development in the first direction does not necessarily mean that the chromatogram is unsatisfactory. In the chromatogram illustrated in Fig. 7 the mixture formed a streak after development in the first direction. However, the apparent streaking was due to overlapping spots which have been separated by the development in the second direction.

Fig. 8 shows a separation of a mixture containing *n*-alkanal $C_{4,7,10}$ (S4, S7, S10), *n*-alk-2-enal $C_{5,8,11}$ (E5, E8, E11) and *n*-alka-2,4-dienal $C_{7,11,14}$ (D7, D11, D14) 2,4-dinitrophenylhydrazones. The adsorbent is the aluminium oxide G–$AgNO_3$ mixture described in Section II B. The plate was heated at 115–135° for 20 min and left overnight as described above. The chromatogram was developed once to the edge of

the plate with 16 % diethyl ether in light petroleum (b.p. 30–40°). The plate was then impregnated with phenoxyethanol as described in the preceding paragraph and developed in the second direction with light petroleum (b.p. 100–120°, not aromatic-free). A satisfactory separation was obtained in this case after the solvent had ascended 14 cm from the starting line.



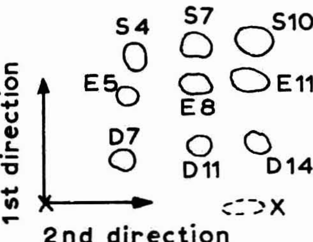


Fig. 8. Two-dimensional separation of normal 2,4-dinitrophenylhydrazones of butanal (S4); heptanal (S7); decanal (S10); pent-2-enal (E5); oct-2-enal (E8); undec-2-enal (E11); hepta-2,4-dienal (D7); undeca-2,4-dienal (D11); tetradeca-2,4-dienal (D14). (X is an impurity). Adsorbent: aluminium oxide G containing 20 % $AgNO_3$ (w/w). Solvent: *first direction* 16 % diethyl ether in light petroleum (b.p. 30–40°) allowed to travel to the edge of the plate once; *second direction* plate dipped in 10 % phenoxyethanol in acetone and developed once with light petroleum (b.p. 100–120°) 14 cm from start to front.

The rate of blackening of spots cannot be used for further identification of classes when the plates have been impregnated with phenoxyethanol because under these conditions the spots tend to fade rather than blacken.

The two chromatograms shown in Figs. 6 and 7 illustrate the separation of mixtures containing either *n*-alkanals, *n*-alkan-2-ones and *n*-alk-1-en-3-ones or only aldehydes of different degrees of unsaturation. To identify a mixture containing members of all these classes the following procedure should be adopted.

1. The unknown mixture is examined two-dimensionally on an alumina plate. This will identify the ketones except acetone which may form one spot with lower aldehydes. This chromatogram will also give an indication of the relative concentration of the various components as well as the carbon number range of the aldehydes present. There may also be some separation between *n*-alkanals and unsaturated aldehydes, the unsaturated aldehydes having a slightly lower R_F value in the first direction. The phenoxyethanol does not interfere with the intense red colour for monocarbonyl 2,4-dinitrophenylhydrazones produced by spraying with alcoholic NaOH.

2. One or more spots are developed on an aluminium oxide G plate with 4 % ether in light petroleum (b.p. 30–40°). A clear separation between aldehydes and

ketones is required at this stage and the plate may therefore have to be developed twice. The areas corresponding to aldehydes are scraped off the plate with a spatula and the aldehydes are extracted from the adsorbent with diethyl ether. The extract is evaporated to a small volume and rechromatographed two-dimensionally on an aluminium oxide G–$AgNO_3$ plate. This will identify the aldehydes according to degree of unsaturation, configuration and position of double bond and carbon number. If acetone is present in the original mixture it will travel with or just ahead of the higher alka-2,4-dienals and propenal in the first direction. However, it travels ahead of propenal and behind the higher alka-2,4-dienals in the second direction.

3. A mixture is prepared containing all the compounds tentatively identified above and mixed in approximately equal proportions with the unknown mixture. Steps 1 and 2 are then repeated with this mixture: if the same number of spots as previously is obtained on both two-dimensional chromatograms, the compounds in the unknown mixture have been identified with the degree of certainty which any chromatographic procedure affords. The possibility of the presence of members of other homologous series of 2,4-dinitrophenylhydrazones has not been excluded.

DISCUSSION

The most comprehensive system for the separation of 2,4-dinitrophenylhydrazones so far published is that of GADDIS AND ELLIS[16] who used adsorption chromatography on paper to separate 2,4-dinitrophenylhydrazones into homologous series of normal alkan-2-ones, alkanals, alk-2-enals and alka-2,4-dienals, and various partition systems with impregnated paper to separate individual members of homologous series. They state that their systems will not separate the pairs acetone–propanal, acetone–butanal and ethanal–propenal. These pairs are separated on the Al_2O_3–$AgNO_3$ system. The present author also found that vinyl ketones run with the methyl ketones in the GADDIS AND ELLIS adsorption system whereas these series readily separate on alumina plates.

SMITH AND OHLSON[17] have discussed the factors which influence retention time in the gas chromatographic separation of unsaturated hydrocarbons using $AgNO_3$ in ethylene glycol as the stationary phase. The same considerations apparently apply in TLC of 2,4-dinitrophenylhydrazones of saturated and unsaturated aldehydes and ketones. R_F values of aldehyde 2,4-dinitrophenylhydrazones decrease with increasing number of double bonds, consistent with increased stability of the silver complex[14]. Nona-2,4-dienal 2,4-dinitrophenylhydrazone has a higher R_F value than the nona-2,6-dienal derivatives as would be expected from the reported lower stability of the silver complexes of conjugated olefins as compared with unconjugated olefins[14]. The separation between the *trans,trans*- and the *trans,cis*-isomers of nona-2,6-dienal 2,4-dinitrophenylhydrazone is also in accord with the easier formation of complexes with *cis*- rather than *trans*-double bonds[15]. This fact has recently been used in the very elegant separation of methyl elaidate (the *trans*-isomer) and methyl oleate (the *cis*-isomer) on a column of silica gel impregnated with $AgNO_3$, the *trans*-isomer having a lower retention volume than the *cis*-isomer[18].

It is to be expected that compounds, other than $AgNO_3$, which are also known to form complexes with olefinic linkages should also prove effective in the separation of compounds according to number, position and configuration of double bonds,

e.g. I_2[19], $AlCl_3$, $FeCl_3$, $ZnCl_2$, CuCl, $Hg(OAc)_2$[14] or any compounds known to act as Lewis acids, and transition metals such as Pt, Pd, Rh[20] etc. Indeed $Hg(OAc)_2$ has been used for this purpose. MANGOLD[21] describes the use of $Hg(OAc)_2$ for the separation of lipids into classes according to degree of unsaturation and configuration of double bond and PREY, BERGER AND BERBALK[22] use $Hg(OAc)_2$ adducts for the separation of olefins. Several column chromatographic procedures are described in the literature for the separation of 2,4-dinitrophenylhydrazones into classes. Although none of these authors offer any explanation for the effectiveness of their methods, complex formation could easily play a part in some of them. VAN DUIN[23] used $ZnCO_3$ columns to separate 2,4-dinitrophenylhydrazones into alkan-2-one, alkanal, alk-2-enal, and alka-2,4-dienal derivatives by displacement with a mixture of light petroleum–benzene (9:1) to which varying quantities of pyridine were added. BADINGS[24] adapted this method to TLC for the separation of oct-2-enal and deca-2,4-dienal 2,4-dinitrophenylhydrazones. SCHWARTZ, PARKS AND KEENEY[25] separated aliphatic monocarbonyls into classes on magnesia–Celite columns. On their columns the formaldehyde and acetaldehyde derivatives travelled with the higher 2-enals, acrolein with the higher 2,4-dienals, and penta-2,4-dienal had a distinctly greater retention volume than the higher 2,4-dienals. This behaviour is paralleled on Al_2O_3–$AgNO_3$ plates (Fig. 6) where acetaldehyde 2,4-dinitrophenylhydrazone has the same R_F value as the higher 2-enal derivatives, acrolein 2,4-dinitrophenylhydrazone travels with the 2,4-dienal derivatives and penta-2,4-dienal has a considerably lower R_F value than the higher 2,4-dienal derivatives.

It is also possible that complex formation between metal ions of the paper and unsaturated 2,4-dinitrophenylhydrazones plays a part in the class separation of GADDIS AND ELLIS[16] and the acetylated paper system of FORSS AND RAMSHAW[9]. This is further supported by the marked effect of temperature on these separations. At lower temperatures complex formation is favoured[14] and hence streaky spots are produced on acetylated paper[9], whereas at higher temperatures, which are less favourable to complex formation, the spots are more compact.

SUMMARY

Thin-layer chromatographic procedures are described for the separation of 2,4-dinitrophenylhydrazones into:

1. Individual members of homologous series by a partition system between 2-phenoxyethanol supported on kieselguhr G and light petroleum (b.p. 100–120°),

2. *n*-Aldehyde, *n*-alkan-2-one and *n*-alk-1-en-3-one derivatives on aluminium oxide G with 4 % diethyl ether in light petroleum (b.p. 30–40°) as the solvent,

3. *n*-Alkanal, *n*-alk-2-enal, *n*-alka-2,4-dienal, *n*-nona-*trans*-2,*trans*-6-dienal and *n*-nona-*trans*-2,*cis*-6-dienal derivatives on plates of aluminium oxide G containing 25 % $AgNO_3$ (w/w) with 16 % diethyl ether in light petroleum (b.p. 30–40°) as the developing solvent.

These procedures have been combined in two-dimensional techniques to give a separation of mixtures of the 2,4-dinitrophenylhydrazones of the normal homologous series of alkan-2-ones, alk-1-en-3-ones, alkanals, alk-2-enals, alka-2,4-dienals, and alka-2,6-dienals.

REFERENCES

[1] K. ONOE, *J. Chem. Soc. Japan, Pure Chem. Sect.*, 73 (1952) 337; from *C. A.*, 47 (1953) 3757d.
[2] J. H. DHONT AND C. DE ROOY, *Analyst*, 86 (1961) 74.
[3] J. ROSMUS AND Z. DEYL, *J. Chromatog.*, 6 (1961) 187.
[4] H. P. KAUFMANN AND Z. MAKUS, *Fette, Seifen, Anstrichmittel*, 62 (1960) 1014.
[5] D. A. FORSS AND W. STARK, *Anal. Chem.*, in preparation.
[6] W. S. LYNN, JR., LOIS A. STEELE AND E. STAPLE, *Anal. Chem.*, 28 (1956) 132.
[7] R. ELLIS, A. M. GADDIS AND G. T. CURRIE, *Anal. Chem.*, 30 (1958) 475.
[8] F. KLEIN AND K. DE JONG, *Rec. Trav. Chim.*, 75 (1956) 1285.
[9] D. A. FORSS AND E. H. RAMSHAW, *J. Chromatog.*, 10 (1963) 268.
[10] H. P. LENK, *Z. Anal. Chem.*, 184 (1961) 107.
[11] D. A. FORSS, E. A. DUNSTONE AND W. STARK, *Australian J. Chem.*, 13 (1960) 584.
[12] E. F. L. J. ANET, *J. Chromatog.*, 9 (1962) 295.
[13] C. B. BARRETT, M. S. J. DALLAS AND F. B. PADLEY, *Chem. Ind. (London)*, (1962) 1050.
[14] S. WINSTEIN AND H. J. LUCAS, *J. Am. Chem. Soc.*, 60 (1938) 836.
[15] P. L. NICHOLS, JR., *J. Am. Chem. Soc.*, 74 (1952) 1091.
[16] A. M. GADDIS AND R. ELLIS, *Anal. Chem.*, 31 (1959) 870.
[17] B. SMITH AND R. OHLSON, *Acta. Chem. Scand.*, 16 (1962) 351.
[18] B. DE VRIES, *Chem. Ind. (London)*, (1962) 1049.
[19] J. G. TRAYNHAM AND J. R. OLECHOWSKI, *J. Am. Chem. Soc.*, 81 (1959) 571.
[20] G. N. SCHRAUZER AND S. EICHLER, *Chem. Ber.*, 95 (1962) 260.
[21] H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.
[22] V. PREY, A. BERGER AND H. BERBALK, *Z. Anal. Chem.*, 185 (1962) 113.
[23] H. VAN DUIN, *Neth. Milk Dairy J.*, 12 (1958) 74.
[24] H. T. BADINGS, *J. Am. Oil Chemists' Soc.*, 12 (1959) 648.
[25] D. P. SCHWARTZ, O. P. PARKS AND M. KEENEY, *Anal. Chem.*, 34 (1962) 669.

SHORTENING OF DEVELOPMENT TIME IN THIN-LAYER ADSORPTION CHROMATOGRAPHY

APPLICATION TO THE SEPARATION OF STEROIDS

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One of the greatest advantages of thin-layer adsorption chromatography, described by STAHL[1], is the significant shortening of the time required for complete chromatographic analysis. With Merck's Silica Gel G, the time of development of thin-layer chromatograms varied from 20 to 120 min depending on the composition of the elution mixtures[1,2].

In our method of partition thin-layer chromatography[3], in which Celite 545 and Zaffaroni's solvent systems were used, the time of development was shortened to 3–7 min. Nevertheless the necessity for evaporating the slightly volatile components of Zaffaroni's stationary phases (formamide, glycols), prior to detection, prolonged the time of complete analysis.

In our present work we used mixtures of Silica Gel and Kieselguhr for coating the plates for thin-layer adsorption chromatography and were successful in effectively shortening the time required for complete chromatographic analysis. The addition of Kieselguhr to active adsorbents in order to increase the rates of flow from chromatographic columns has been previously described[4]*.

We used Merck's Silica Gel G or Light's Silica Gel, specially prepared, (see Experimental) both mixed with 20 % or 50 % of Celite 545. Chromatograms were developed with various elution mixtures and the times taken by the front to move 10 cm were observed. The results are shown in Table I.

As can be seen, the addition of an equal amount of Celite 545 to Light's Silica Gel increased the rate of travel of the elution front about 3.5 times. In the case of Merck's Silica Gel G the increase was 2.5 times. Depending on the composition of elution mixtures, the time of development varied from 3–15 min (in one case only it was equal to 19 min).

As the elution mixtures commonly used in thin-layer adsorption chromatography consist mainly of low-boiling organic solvents, the time of drying is very short, *i.e.* 2–4 min and the complete chromatographic analysis can be carried out in about 20 min. It is particularly useful in cases of chemical or enzymic kinetic studies as for instance in biosyntheses of steroids.

In the bio-oxidation reaction of MAMOLI AND VERCELLONE[6] where 3,21 diacetoxy-

* Quite recently BENNETT AND HEFTMANN[5], have used plates coated with mixtures of Silica Gel G and Kieselguhr G (1:1) for better separation of C-25 sapogenins.

TABLE I

TIMES TAKEN BY THE LIQUID FRONT TO MOVE 10 CM FOR DIFFERENT ELUTION MIXTURES AND DIFFERENT ADSORPTION LAYERS

Elution mixture	*Adsorption layer*					
	Specially prepared Light's Silica Gel			*Merck's Silica Gel G*		
	No Celite added	*With Celite 545*		*No Celite added*	*With Celite 545*	
		4:1	*1:1*		*4:1*	*1:1*
Cyclohexane	54	38	19	32	29	15
Cyclohexane–benzene (1:1)	36	27	14	25	20	14
Toluene	33	27	11	20	16	9
Benzene	43	27	11	22	16	10
Toluene–ethyl acetate (9:1)	35	22	10	21	14	8
Toluene–ethyl acetate (1:1)	37	23	11	20	14	8
Ethyl acetate	31	23	11	20	17	8
Chloroform–ethyl acetate (1:1)	34	21	15	25	22	10

Δ^5-pregnen-17-ol-20-one was transformed into Reichstein "Substance S", using a mixture of Merck's Silica Gel G and Celite 545, we were able to give the semiquantitative results 35 min after taking a sample from the fermenter.

The addition of Celite 545 to the adsorbents changed only to a small extent the selectivity of the resolution of steroidal mixtures. The R_F values were increased, as we expected, because of the dilution of the active adsorbent in the layer.

A comparison of two chromatograms obtained by different methods is presented in Table II and Fig. 1.

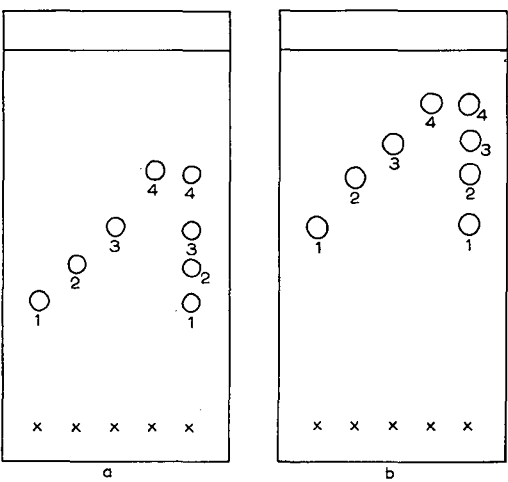

Fig. 1. Schematic view of the chromatograms described in Table II. Spot numerals correspond to the numerals of steroid substances in Table II. 30 γ of each steroid substances was applied. Detection with isonicotinic hydrazide. (a) Adsorbent Merck's Silica Gel G. Average diameter of spot 5 mm. Time of development 16–19 min. (b) Adsorbent mixture (1:1) of Merck's Silica Gel G and Celite 545. Average diameter of spot 6 mm. Time of development *ca.* 8 min.

TABLE II

COMPARISON OF THE R_F VALUES OF SOME STEROIDS ON THIN LAYERS OF SILICA GEL G AND A MIXTURE (1:1) OF SILICA GEL G AND CELITE 545

Elution mixture = Benzene–ethyl acetate (1:1)

No.	*Steroid*	*Thin layer of Silica Gel G*	*Thin layer of a mixture of Silica Gel G and Celite 545 (1:1)*
1.	Δ^1-Dehydromethyltestosterone	0.33	0.52
2.	Methyltestosterone	0.43	0.65
3.	Androstendion	0.53	0.74
4.	Methyltestosterone acetate	0.68	0.85

EXPERIMENTAL

(1) *Preparation of adsorbents*

Celite 545 (L. Light & Co, England) was ground in a ball mill and sifted through a sieve DIN 1171, 0.071 mm. Silica Gel (100/200 mesh) for chromatography (L. Light & Co, England) was ground in a ball mill and sifted through a sieve DIN 1171, 0.071 mm.

(2) *Preparation of plates*

Three plates of mirror glass (thickness 5 mm, width 100 mm, length 180 mm) were coated with a slurry of given alternative composition:

(a) 3.5 g of Silica Gel prepared as described in (1); 3.5 g of Celite prepared as described in (1); 0.4 g of gypsum sifted through a sieve DIN 1171, 0.071 mm; 20 ml of water.

(b) 3.5 g of Merck's Silica Gel G; 3.5 g of Celite 545 prepared as described in (1); 20 ml of water.

The slurry was spread on the plates by means of a glass rod. Uniform layers were afterwards obtained by shaking the plates by hand; the plates were dried horizontally in an oven while the temperature was raised gradually from 20° to 120° over a period of one h.

Comparative plates with pure adsorbents were made in a similar manner. The coating slurries were of the following compositions:

(c) 7 g of Silica Gel prepared as described in (1); 0.4 g of gypsum sifted through the sieve DIN 1171, 0.071 mm; 20 ml of water.

(d) 7 g of Merk's Silica Gel G; 20 ml of water.

SUMMARY

The application of mixtures (1:1) of Silica Gel and Celite 545 to adsorption thin-layer chromatography shortened the time of development 2.5 to 3.5 times as compared to chromatograms in which the pure absorbents were used. The addition of Celite to Silica Gel increased the R_F values but it had no influence on the selectivity of the steroid mixture resolution.

REFERENCES

1 E. STAHL, *Z. Anal. Chem.*, 181 (1961) 303.
2 E. DEMOLE, *J. Chromatog.*, 6 (1961) 2.
3 J. VAEDTKE AND A. GAJEWSKA, *J. Chromatog.*, 9 (1962) 345.
4 E. LEDERER AND M. LEDERER, *Chromatography*, 2nd Ed., Elsevier, Amsterdam, 1957, p. 24
5 R. D. BENNETT AND E. HEFTMANN, *J. Chromatog.*, 9 (1962) 353.
6 L. MAMOLI AND A. VERCELLONE, *Ber.*, 71 (1938) 1686.

THE PAPER CHROMATOGRAPHY OF STEROID ESTERS

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Chromatographic procedures for the separation of steroids are now well established and have been the subject of reviews by BUSH[1], HEFTMANN[2], NEHER[3] AND EDWARDS[4]. Di-ketones, di-esters and keto-esters of the steroids generally move with the solvent front and, until recently, have only been adequately resolved by reversed phase partition techniques. KRITCHEVSKY AND CALVIN[5] treated the paper with Quilon (stearato-chromic chloride) and KRITCHEVSKY AND TISELIUS[6] treated it with silicone.

NEHER AND WETTSTEIN[7] impregnated the paper with phenyl cellosolve and eluted with hexane in a non-reversed phase procedure but this, like the reversed phase methods, leads to gross contamination of the steroids when recovering them unchanged after chromatography.

ZANDER AND SIMMER[8] modified the solvent mixture of BUSH[9] and obtained improved separations, illustrated by the R_F** change for progesterone from 91 to 72. This type of solvent mixture, petroleum or ligroin with aqueous methanol, is ideal when recovery of the steroid is required and the procedure involved is capable of completion in much shorter times than are the previously described methods. EDWARDS[4] showed that the resolution could be improved by employing partition between anhydrous methanol and petroleum at 4°, giving an R_F of 25 for progesterone and an effective separation of esters.

The resolution of these relatively "low-polar" steroid derivatives is further improved in the partition between formic acid and light petroleum recently reported in preliminary form[10] and now to be fully described.

EXPERIMENTAL

The general procedures of steroid chromatography are described elsewhere[4] and only special points will be elaborated.

Solvents

Formic acid, A.R.; light petroleum, b.p. 80–100°, A.R.; methanol and benzene were used as supplied.

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** R_F is expressed as the percentage fraction of the distance moved by the solvent front.

Apparatus

(1) The "Universal Chromatography Apparatus" described by SMITH[11] and supplied by Messrs. Aimer Products, 56 Rochester Place, London, N.W. 1, was used. The Datta frame carries five papers in a vertical position for ascending chromatography. For the equilibration it is suspended by cross wires and a rod (Fig. 1) and then lowered into the solvent in the tray for the chromatographic run.

(2) Cylindrical jar (30 cm high, 10 to 15 cm diam.) and lid with central hole (7 mm diam.).

Paper

Whatman grades numbered 1, 2, 4 and 3MM supplied in 25 cm squares with corner holes are assembled into the frame or sewn into cylinders by cotton thread and used in the jars.

Steroids

Reference steroids were commercial products or were kindly supplied by Dr. W. KLYNE from the M. R. C. Reference steroid collection. Formates were prepared on an

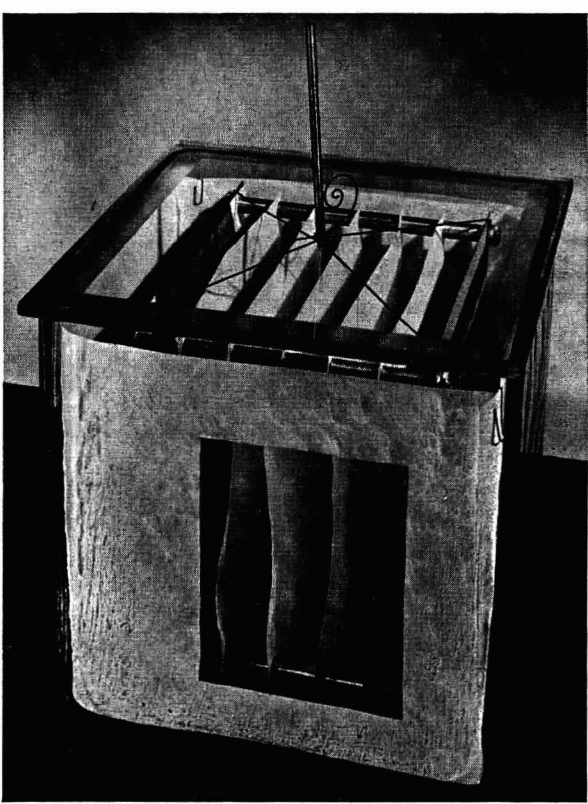

Fig. 1. The "Universal Chromatography Apparatus". The frame is shown in suspended position for equilibration. The curled pin is removed and the frame lowered into the mobile phase to start ascent of solvent.

approximate 100 μg scale by standing the steroids in formic acid solution overnight. The excess acid was evaporated in a stream of nitrogen at 40°; no further purification was employed. Acetates and propionates were prepared by treating 100 μg quantities of steroid with the respective anhydride (0.2 ml) and pyridine (0.2 ml). After standing overnight the solvents were removed in a stream of nitrogen at 40°, assisted when necessary by the additions of small volumes of toluene.

Location of steroids on chromatograms

Steroids were located[4] by use of characteristic physical and chemical properties, including the absorption of 254 mμ ultraviolet light, reaction with alkaline *m*-dinitrobenzene and reduction of phosphomolybdic acid.

Chromatography

The steroids to be chromatographed were placed on origins 2 cm apart along a line drawn 4 cm from one edge of the 25 cm square paper and elution was effected by the ascending solvent by one of two procedures.

(*1*) *The Universal Apparatus.* Up to five papers were mounted in the frame, separated from one another by dural collars. Between the outer collars and the side plates of the frame were placed small paper "washers" to absorb condensation from the metal. Two cross wires were fixed to the top four corner nuts and these wires passed through holes near one end of a brass rod (6 mm diam.) at the point where they crossed, and by this means the frame could be suspended freely by lifting the rod (Fig. 1).

The walls of the Universal Chromatography tank were lined with filter paper (3MM), except for a window cut to permit observation of the movement of the solvent front. This paper was soaked with stationary phase (200 ml) placed in the bottom of the tank and surrounding the dural trough charged with mobile phase (200 ml). The rod carrying the frame and papers was passed through the central hole of the lid of the tank and was pinned so that the frame hung, evenly balanced, from the lid when it was raised. On sealing the lid on the tank with silicone grease the chromatogram papers were suspended in position for equilibration. After 4 h at room temperature the chromatographic run was started by lowering the frame into the trough of mobile phase.

(*2*) *The jar chromatographic apparatus.* The jar was lined with paper (3MM) except for an observation window (5 × 5 cm). This paper was soaked with stationary phase (25 ml) placed in the bottom of the jar and surrounding a Petri dish. The chromatogram was sewn into a cylinder (with a 5 mm gap between the meeting edges) by knotted strands of cotton at three evenly spaced intervals along the length. The lid was sealed in position with silicone grease and after 4 h equilibration the mobile phase (10 ml) was added through a long stemmed funnel inserted temporarily through the central hole and which touched the Petri dish.

In each apparatus the ascent of the solvent front to within 1 cm of the top of the papers took about 45 min at room temperature. The papers were then removed and the solvent evapoιated in a stream of air. After location of the steroids the R_F values were calculated and expressed as the percentage fraction of the distance moved by the solvent front.

Solvent mixtures

Formic acid and light petroleum were mixed in the proportions given in Table I with the inclusion of other solvents as required.

TABLE I

R_F VALUES OF STEROIDS AND STEROID ESTER IN FORMIC ACID–LIGHT PETROLEUM MIXTURES

The R_F values of non-esterified alcohols were observed after 0.5 h equilibration at approx. 18°, those of the other steroids after 4 h equilibration.

Designation	*Fo*		*F1*				*F2*	
*Solvents** *Formic acid*	*100*		*100*				*100*	
Light petroleum (80–100°)	*100*		*90*				*50*	
Benzene							*50*	
Methanol			*10*					
*Steroid***	*Free*	*Ft*	*Free*	*Ft*	*Ac*	*Pr*	*Free*	*Ft*
Cholestenone	96		98					
Oestrone								22
Androstane derivatives								
5α-Androstan-17-one	95		97					
Androst-5-en-3β-ol		93		93				
5α-Androstane-3α,17β-diol		77		84				87
5β-Androstane-3α,17β-diol		78		88				89
Androst-5-ene-3β,17α-diol		76		86				89
Androst-5-ene-3β,17β-diol		78		85				89
17α-Methyl-5α-androstane-3β,17β-diol	11	48		66				82
17α-Methyl-androst-5-ene-3β,17β-diol		48		55				79
5α-Androstan-3β-ol-17-one		25		54	62	77		68
5α-Androstan-3α-ol-17-one	7.5	32	11	51	59	78	55	68
5β-Androstan-3α-ol-17-one	5.5	29	8	52	57	73	31	74
5β-Androstan-3β-ol-17-one	6	30					47	82
5β-Androst-9(11)-en-3α-ol-17-one	7.5	32	11	58	64	81	51	76
Androst-5-en-3β-ol-17-one	5	28	9	51	56	74	29	72
5β-Androstan-17β-ol-3-one	5	25	9	43				75
Androst-4-en-17β-ol-3-one		8	3	20	23	42	16	49
Androst-4-en-17α-ol-3-one		8	3	19	20	39	16	49
Androsta-[illegible],4-dien-17β-ol-3-one								29
17α-Methylandrost-4-en-17β-ol-3-one	0		6		32		25	
19-Nor-androst-4-en-17β-ol-3-one		8		16				48
5α-Androstane-3,17-dione	5		13				46	
5β-Androstane-3,17-dione	5		13				45	
Androst-4-ene-3,17-dione	3		3				20	
Androsta-1,4-diene-3,17-dione							10	
Androst-4-ene-3,11,17-trione			2				24	
Androst-4-en-11β-ol-3,17-dione			0				2	
5β-Androstane-3α,11β-diol-17-one				3				27
Androst-4-ene-11β,17β-diol-3-one				2				13
Pregnane derivatives								
5α-Pregnan-3β-ol		91		93				
5β-Pregnan-3β-ol		83		93				
5α-Pregnane-3α,20α-diol		86		88				93
5β-Pregnane-3α,20α-diol		86		88				94
Pregn-5-ene-3β,20α-diol		85		88				92
Pregn-5-ene-3β,20β-diol		84		89				93
5α-Pregnane-3α,17α,20α-triol		23		36				70

(continued on p. 216)

TABLE I (*continued*)

Designation	*Fo*		*F1*				*F2*	
*Solvents**								
Formic acid	*100*		*100*				*100*	
Light petroleum (80–100°)	*100*		*90*				*50*	
Benzene							*50*	
Methanol			*10*					
*Steroid***	*Free*	*Ft*	*Free*	*Ft*	*Ac*	*Pr*	*Free*	*Ft*
5β-Pregnane-3β,16β,20α-triol		22						73
5α-Pregnan-3α-ol-20-one		70	35	76				86
5β-Pregnan-3α-ol-20-one		65	28	77				89
Pregn-4-en-20β-ol-3-one		19		28	33	52		72
Pregn-5-en-3β-ol-20-one		61	23	76	80	86	60	86
Pregna-5,16-dien-3β-ol-20-one							58	86
5α-Pregnane-3,20-dione	19		36				68	
5β-Pregnane-3,20-dione	19		36				68	
Pregn-4-ene-3,20-dione	4		11				48	
Pregna-4,16-diene-3,20-dione							49	
16α-Methyl-pregn-4-ene-3,20-dione							62	
Pregn-4-ene-3,11,20-trione			0				8	

* Top phase is mobile phase.
** Ft = formate; Ac = acetate; Pr = propionate; mono-, di- or tri-ester as appropriate.

RESULTS

Many solvent mixtures were tried but the three given in Table I were extensively investigated because of the separation of the three more important groups of steroid derivatives.

Formylation of 3α, 3β, 11α, 17α- and 17β-androstane, 20α and 20β hydroxyl groups was observed during equilibration since, with longer equilibration, a substance of low mobility on the chromatogram was replaced by a substance of higher mobility which possessed the same R_F as the formate prepared independently. 11β- and 17α-pregnane hydroxyls were not esterified. When acetates or propionates were chromatographed there was no evidence of exchange of formoxy- for acetoxy- or propoxy-groups.

Reliability of the chromatographic procedure

R_F values of reference steroids in a given solvent mixture were constant to within 3 units and independent of, (1) position on the paper, (2) weight of the paper (*i.e.* Whatman grade 1, 2, 4 or 3 MM), (3) position of paper in the frame, (4) type of apparatus employed (Universal apparatus or jar) and, (5) occasion of run.

Effect of variation of solvent constitution

Substitution of lower or higher boiling grades of light petroleum or ligroin for the 80–100° grade had little effect except that "tailing" occurred with the highest boiling fractions.

Load of steroid

When portions progesterone from 100 to 2000 μg were applied to origins of a single paper and run in the F2 system, little enlargement of the spots occurred up to 500 μg/cm². At 1000 μg/cm² spot diameter increased by 50% and at twice this oad the diameter was nearly trebled.

DISCUSSION

The procedures described facilitate the paper chromatography of those androstane and pregnane derivatives which possess two weakly polar substituents, such as ketones or esterified alcohols. The 4-h equilibration prior to chromatography is the time required for formylation of the unesterified hydroxyl groups. The 11β-hydroxyl and the 17α-hydroxy-pregnane are the anticipated exceptions since these are hindered groups of the type discussed by KLYNE[12]. This condition of esterification is mild and similar to that of formylation in formic acid solution at room temperature (pyridine catalyst is not required).

Whilst the use of the light petroleum and formic acid mixture was suitable for the chromatography of the esters of dihydroxy-steroids, addition of methanol or benzene was necessary for the esters of mono-ketonic alcohols and the diketones.

Pairs of isomeric steroids were separated to a lesser or greater degree. Thus, whilst epimeric 3, 5 (including -5-ene-), 17 and 20 substituents were not well resolved, if at all, larger scale isomers were resolved as shown by the separation of the groups of androstan-3-on-17-ols and androstan-17-on-3-ols.

The effect of substituents tended to be much greater, particularly that of the polar groups, such as the 11-ketone and 11-hydroxyl. A somewhat smaller but still powerful polar effect was associated with the addition of a -4:5- double bond in conjugation with a -3-ketone, as illustrated by the decrease of R_F by 23 units when testosterone was formed. No such change was associated with the -16:17- double bond in conjugation with a 20-ketone (from pregnenolone). The isolated -9:11- double bond when added to aetiocholanolone produced an R_F increase of 6 units. This decreased polarity must be explained by an association with benzene of the mobile phase, rather than with formic acid of the stationary phase.

The difference of property of pregnane derivatives in comparison to otherwise similar androstane derivatives can be considered as due to a substituent, that of the two carbon side chain. It is possible that other changes in the carbon skeleton would have similar marked effects but the following few examples are hardly enough for generalisation.

Addition of a methyl group has an effect, since 16α-methyl-progesterone is considerably less polar than progesterone and testosterone is less polar than its 19-nor-analogue. The effect of the 17α-methyl substituent in the androstane series is masked by the concomitant change of the 17β-hydroxyl to tertiary character, but 17β-acetoxy-methyl-testosterone is less polar than testosterone acetate.

Esterification had the most pronounced effect since the free steroids have very much lower R_F values than the esters. Each esterifying group had a different effect, polarity decreasing in the order formate, acetate, propionate. Such esterification had a much greater effect on R_F than a corresponding change to a ketone which results in an inversion of the order of separation of the major groups, diols, ketols and diones, when comparing the formic acid chromatography with procedures for free steroids, such as light petroleum–methanol partition[9].

Application of the light petroleum and formic acid mixtures to the chromatography of steroids of a higher degree of oxygenation and to that of crude mixtures of biological origin is in hand. Valuable results are expected, particularly since the steroids and their esters are highly soluble in both phases of the solvent mixture.

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SUMMARY

1. Two phase solvent mixtures are described in which di-substituted steroid ketones and esters have partition coefficients such that they may be readily separated by partition chromatography on paper.

2. 80 steroids and derivatives have been characterised in these solvent systems and their R_F values are presented. Notable features include the resolution of otherwise similar androstane and pregnane derivatives, 3-hydroxy-17-ketone from 17-hydroxy-3-ketones in the androstane series, aetiocholanolone from its -9:11- unsaturated analogue and substantial inversion of general order of separation as compared with the order observed in procedures for unesterified steroids.

3. Up to 0.5 mg of steroid or derivative per cm^2 may be chromatographed from each origin.

REFERENCES

1 I. E. BUSH, *Brit. Med. Bull.*, 10 (1954) 229.
2 E. HEFTMANN, *Chem. Rev.*, 55 (1955) 679.
3 R. NEHER, *Chromatographic Reviews*, Vol. 1, Elsevier, Amsterdam, 1959, p. 99.
4 R. W. H. EDWARDS, in I. SMITH, *Chromatographic and Electrophoretic Techniques*, 2nd Ed., Heinemann, London, 1960, pp. 409 and 421.
5 D. KRITCHEVSKY AND M. CALVIN, *J. Am. Chem. Soc.*, 72 (1950) 4330.
6 D. KRITCHEVSKY AND A. TISELIUS, *Science*, 114 (1951) 299.
7 R. NEHER AND A. WETTSTEIN, *Helv. Chim. Acta*, 35 (1952) 276.
8 J. ZANDER AND H. SIMMER, *Klin. Wochschr.*, 32 (1954) 529.
9 I. E. BUSH, *Biochem. J.*, 50 (1952) 370.
10 R. W. H. EDWARDS, *Biochem. J.*, 74 (1960) 27P.
11 I. SMITH, *Chromatographic and Electrophoretic Techniques*, 2nd Ed., Heinemann, London, 1960.
12 W. KLYNE, *The Chemistry of the Steroids*, Methuen, London, 1957, p. 71.

A VERSATILE SOLVENT TO REPLACE PHENOL FOR THE PAPER CHROMATOGRAPHY OF RADIOACTIVE INTERMEDIARY METABOLITES

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Analysis of the products of radioactive tracer feeding experiments in biological systems has depended to a considerable extent on paper chromatography. Of the many solvent systems proposed, the one devised by BENSON *et al.*[1], based on the use of phenol–water followed by *n*-butanol–propionic acid–water, has many advantages for the separation of such biological intermediates as sugars, sugar phosphates, amino acids and carboxylic acids. Nevertheless, phenol–water mixtures have several inherent drawbacks; MIZELL AND SIMPSON[2] recently surveyed some of these, and to this list we might add both the corrosive action of phenol on human skin and the fact that many amino acids show signs of decomposition when chromatographed in phenol-containing solvents[3,4].

The quest for a solvent to replace phenol led us to consider some of those mentioned in the literature[2,5,6], but none were suitable for our purpose. Eventually interest centered on solvents based on isobutyric acid, ammonia, water and ethylenediaminetetraacetic acid (EDTA)[7]. Although this solvent was not entirely satisfactory as originally formulated[7], because such slow-running hydrophilic substances as sugar phosphates migrated too far and left empty much of the chromatogram near the origin, the mixture seemed promising as a basis for further experimentation. There were two approaches towards improving both resolution and the spread of compounds along the whole length of the chromatogram: manipulation of the pH, and an enhancement of the non-aqueous character of the liquid. The pH of the original isobutyric acid–ammonia–water–EDTA solvent is about 4.2; replacement of some of the water with sufficient ammonia solution to raise the pH to 6.8 resulted in a relatively viscous and very slow-running solvent in which the resolution and spread actually deteriorated.

The first attempts to reduce the aqueous nature of the solvent consisted of replacing the ammonia solution, partially or completely, with such organic bases as pyridine and trimethylamine in various combinations, always maintaining the pH at 4.2. A further variation was to saturate the original solvent mixture with toluene. It soon became apparent that the use of these substances did little to improve the chromatographic separation; however, we were stimulated to invent quite a complex vocabulary to describe the olfactory impact of the products. Subsequent alteration of the solvent mixtures was directed to a partial replacement of the water by one of

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a number of simple alcohols: ethanol, *n*-propanol, isopropanol and *n*-butanol. This led to marked improvements in solvent characteristics, though no single alcohol was completely satisfactory in this regard. Mixing of those alcohols which looked promising when used singly was then attempted. After numerous trials, a mixture (hereafter designated "semi-stench") was prepared which possessed the most suitable solvent properties for our purposes.

EDTA	1.2	g
17 *N* Ammonia solution	100	ml
Water	950	ml
n-Propanol	350	ml
Isopropanol	75	ml
n-Butanol	75	ml
Isobutyric acid	2500	ml

On standing at room temperature for 24 h esterification equilibria appeared to be established between the alcohols and isobutyric acid. The ammonium isobutyrate buffered the mixture at a pH of about 4.0. Aging of the mixture, even for a period of weeks, effected no discernible change in resolution properties. On the other hand, a comparison of mixtures aged for at least 24 h with corresponding solutions freshly prepared showed that aging for one day is mandatory for optimal results. No attempt was made to accelerate esterification either by mild heating or by refluxing, since it was quite convenient to prepare the solvent at least a day before use.

For chromatographic development, the two-dimensional descending technique was employed. The semi-stench solvent was used for the first dimension; the second dimension was developed with *n*-butanol–propionic acid–water[1]. Standard Whatman No. 4 chromatography paper was used exclusively; neither oxalic acid-washed paper nor paper rinsed in an alkaline EDTA solution (pH 8.5) was more effective. Both solvents were allowed to run to the edges of the paper, this taking (23°) 10–14 h for the first dimension (*ca.* 50 cm) and 7–10 h for the second (*ca.* 38 cm), depending mainly on the batch of paper. Known compounds were localized by spraying or dipping the paper in the appropriate reagents. Radioactive substances were found by radioautography, positive identification then being made by cochromatography with authentic marker compounds.

In our actual experimental conditions, the compounds of interest are chromatographed in the presence of cell extracts. Since there is probably interaction between compounds in mixtures, the chromatographic parameters of all the compounds investigated were measured by mixing each authentic substance with an ethanol–water extract of *Chlorella pyrenoidosa* cells or of spinach chloroplasts which had been allowed to fix $^{14}CO_2$ photosynthetically[1]. Table I lists the relative R_F values for all the compounds studied with respect to aspartic acid.

The mobilities reported in Table I must be used only as an approximate guide to chromatographic position when compounds are being studied in a complex mixture. These values were obtained by adding two or three known substances at a time to the ^{14}C-labelled plant extract, and relating their position to aspartic acid. We cannot therefore be certain that compounds having nearly identical mobilities will have the precise relationships to each other indicated in Table I, since we have not simultaneously chromatographed all the listed compounds on one sheet of paper. Slight variations between different chromatograms in a series preclude absolute certainty

TABLE I

$R_{aspartic\ acid}$ VALUES IN SEMI-STENCH (A) AND IN *n*-BUTANOL–PROPIONIC ACID–WATER (B)

Compound	$R_{aspartic\ acid}$	
	A	*B*
Amino acids and peptides		
α-Alanine	2.49	1.30
β-Alanine	2.65	1.66
γ-Aminobutyric acid	3.46	2.04
Arginine	2.90	1.14
Asparagine	1.50	1.00
Aspartic acid	1.00	1.00
Citrulline	1.85	1.13
Cysteine	1.01	0.63
Cystine	0.81	0.63
Glutamic acid	1.25	1.08
Glutamine	1.58	0.92
Glutathione, oxid.	0.10	0.55
Glutathione, red.	1.07	0.46
Glycine	1.82	0.96
Histidine	3.66	0.42
Leucine	3.92	2.30
Isoleucine	3.50	2.77
Lysine	2.82	0.67
Methionine	3.23	2.33
Phenylalanine	3.62	2.55
Proline	3.07	1.65
Serine	1.70	0.94
Threonine	2.22	1.20
Tryptophan	2.68	1.77
Tyrosine	2.36	1.64
Valine	3.44	2.61
Carboxylic acids, hydroxy- and oxo-carboxylic acids		
cis-Aconitic acid	0.89	1.48
cis-Aconitic anhydride	0.87	1.76
Citric acid	0.79	1.46
Isocitric acid	0.84	1.57
Fumaric acid	1.13	1.83
Glyceric acid	1.43	1.52
Glycollic acid	1.77	1.73
Glyoxylic acid	1.83	1.55
Lactic acid	2.47	2.16
Malic acid	1.09	1.58
α-Oxoglutaric acid	1.12	1.51
Pyruvic acid	1.75	1.60
Succinic acid	2.00	1.87
Tartaric acid	0.53	0.94
Sugars		
Arabinose	1.53	1.26
2-Desoxyribose	2.65	1.96
Dihydroxyacetone	2.00	2.11
Erythrose	1.71	1.32
Erythrulose	2.19	1.67
Fructose	1.52	1.15
Galactose	1.18	1.02
Glucose	1.20	1.05

(continued on p. 222)

TABLE I (*continued*)

Compound	$R_{aspartic\ acid}$	
	A	*B*
Gulose	1.30	1.07
Lactose	0.63	0.64
Lyxose	1.59	1.29
Maltose	0.74	0.74
Mannose	1.17	1.01
Ribose	1.83	1.36
Ribulose	2.13	1.43
Sedoheptulose	1.21	1.07
Sorbose	1.38	1.13
Sucrose	1.26	0.99
Threose	2.28	1.67
Xylose	1.60	1.34
Xylulose	1.95	1.29
Sugar alcohols		
Arabitol	1.75	1.19
Dulcitol	1.47	1.06
Glycerol	3.03	1.91
Mannitol	1.55	1.14
Ribitol	1.53	1.20
Sorbitol	1.53	1.12
Xylitol	1.72	1.15
Uronic acids		
Glucuronic acid	0.72	0.77
Glucuronolactone	1.67	1.37
Aldonic acids		
Ascorbic acid	1.42	1.69
Gluconic acid	0.78	1.04
Nucleosides		
Adenosine	3.51	1.98
Cytidine	2.54	1.66
Guanosine	1.50	1.37
Inosine	1.42	1.24
Thymidine	2.81	2.47
Uridine	1.67	1.38
Nucleotides		
Adenosine monophosphate	1.35	0.86
Adenosine diphosphate	0.68	0.35
Adenosine triphosphate	0.37	0.23
Guanosine monophosphate	1.35	0.86
Guanosine diphosphate	0.21	0.39
Guanosine triphosphate	0.08	0.22
Inosine monophosphate	0.59	0.49
Thymidine monophosphate	1.27	1.06
Uridine monophosphate	0.91	0.79
Uridine triphosphate	0.10	0.21
Sugar monophosphates		
Dihydroxyacetone phosphate	0.81	0.77
Fructose-6-phosphate	0.55	0.65

TABLE I (*continued*)

Compounds	$R_{aspartic\ acid}$	
	A	*B*
Glucose-6-phosphate	0.42	0.57
Hamamelose-6-phosphate	0.51	0.73
Maltose monophosphate	0.35	0.47
Ribose-5-phosphate	0.68	0.75
Sedoheptulose-7-phosphate	0.42	0.57
Sugar diphosphates		
Fructose diphosphate	0.14	0.24
Hamamelose diphosphate	0.14	0.35
Ribulose diphosphate	0.22	0.39
Miscellaneous phosphates		
Phosphoenolpyruvic acid	0.71	1.16
6-Phosphogluconic acid	0.23	0.71
2-Phosphoglyceric acid	0.60	0.90
3-Phosphoglyceric acid	0.53	0.88
Uridine diphosphoglucose	0.44	0.31

of chromatographic mobility for any particular substance. The mobilities in two dimensions of over twenty compounds was measured with respect to aspartic acid on eight replicate chromatograms similar to the one shown in Fig. 1. The standard deviation of these measurements showed an average for all the spots of $\pm$ 2.8 % of the means in the semi-stench solvent, and $\pm$ 3.9 % of the means in *n*-butanol–propionic acid–water. Thus the values reported in Table I should be regarded as possessing an error of *ca.* 6–8 %.

A radioautogram of a typical chromatogram of ^{14}C-labelled chloroplast extract is shown in Fig. 1, compared with a parallel chromatogram of a similar extract run in phenol–water[1] as the first solvent (Fig. 2). It will be seen that with the semi-stench solvent there is a more effective use than with phenol–water of the total area available on the chromatogram. Both systems have disadvantages resulting from the overlapping of certain compounds which it would be most desirable to have separated. In semi-stench plus butanol–propionic acid this affects mainly glutamine, glycine and serine, while in phenol plus butanol–propionic acid, glutamine runs well clear of the other two amino acids though the latter cannot be separated from glucose. The new system, moreover, results in spots more compact than does phenol, and shows much less variability as far as diffuseness and streaking of the spots is concerned.

In comparing these two solvent systems one effect has consistently been observed, but has not been completely resolved. Using the same cell extract, more spots are separated in the semi-stench than in the phenol system. We think that these are neither artifacts nor degradation products, but reflect the greater resolving power of semi-stench. A preliminary study of the action of semi-stench as far as the decomposition of amino acids is concerned has shown it to be much milder than phenol: the latter causes considerable breakdown of some amino acids[3,4]. The reason for this destruction by phenol is possibly a result of atmospheric oxidation of phenol to form

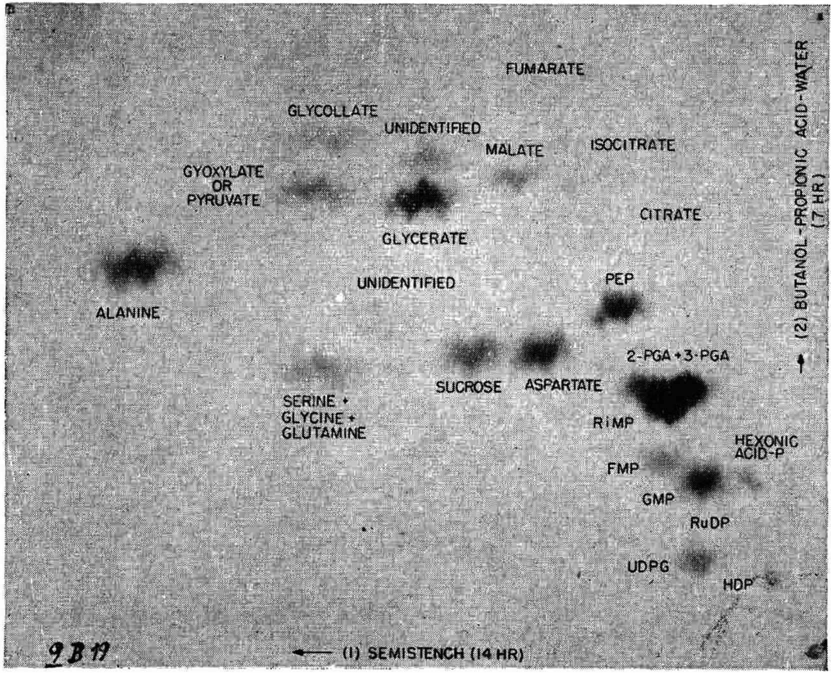

Fig. 1. Radioautograph of chromatogram of extract of ^{14}C-labelled chloroplasts, developed to the edges of the paper only with (1) semi-stench (14 h) and (2) *n*-butanol–propionic acid–water (7 h). FMP = fructose monophosphate; GMP = glucose monophosphate; HDP = glucose and fructose diphosphates; hexonic acid-P = monophosphate of an unidentified hexonic acid; PEP = phoshoenolpyruvic acid; 2-PGA = 2-phosphoglyceric acid; 3-PGA = 3-phosphoglyceric acid; RiMP = ribose monophosphate; RuDP = ribulose diphosphate; UDPG = uridinediphosphoglucose.

the deep red phenoquinone, a relatively strong oxidizing agent. None of the preservatives generally recommended for phenol chromatography completely inhibits the process, though the addition of α-tocopherol or potassium cyanide to the phenol–water mixtures does slow down oxidation[8].

It must be pointed out that some concern may justly be accorded those substances (acid anhydrides, enolic esters, and other high energy compounds) which are often more susceptible to ammonolysis than to hydrolysis. It is possible that some of the newly differentiated spots may be products of ammonolysis of certain of the groups mentioned. While we have neither proven nor disproven this, it should be noted that the versatility of the new solvent system is such that an equimolar quantity of trimethylamine may be substituted for the ammonia without affecting the chromatographic pattern[9].

As with the phenol system, chromatograms developed only to the edges of the paper with semi-stench and butanol–propionic acid show the phosphate esters congregated close to the origin and inadequately separated. Better resolution of these substances may be obtained by over-running the chromatograms for 40–60 h in semi-stench and 20–24 h in butanol–propionic acid. However, in this case, the mobilities of the various phosphates with respect to aspartic acid are not the same as when the chromatograms are developed to the edges of the paper only. Table II lists the

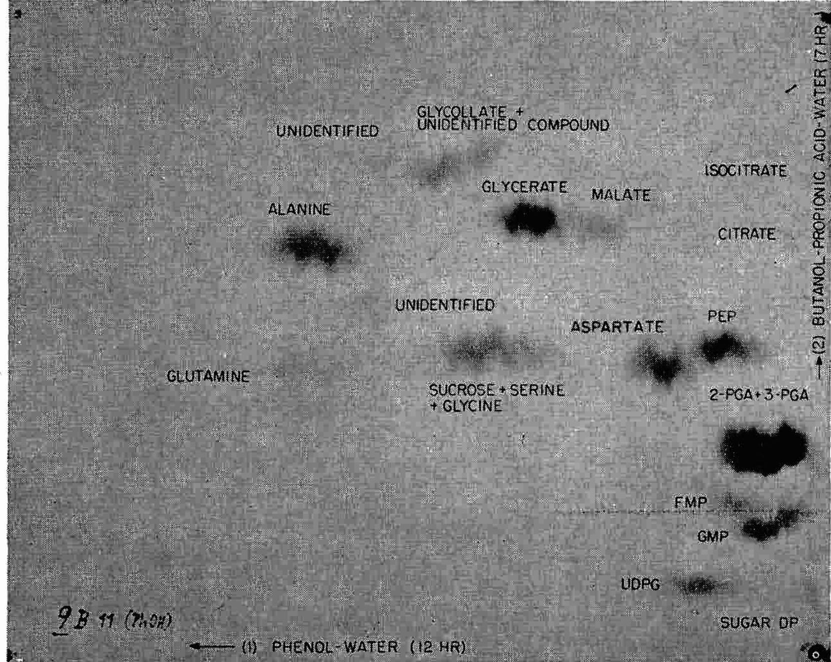

Fig. 2. Radioautograph of chromatogram of the same extract as in Fig. 1, developed to the edges of the paper only with (1) phenol–water (12 h) and (2) *n*-butanol–propionic acid–water (7 h). Sugar DP = sugar diphosphates; other abbreviations as for Fig. 1.

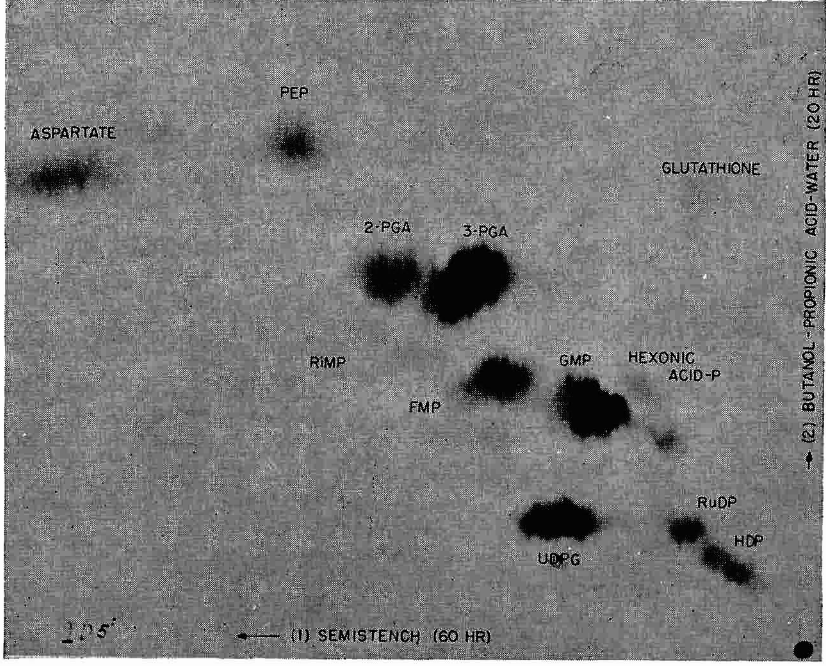

Fig. 3. Radioautograph of chromatogram of the same extract as in Fig. 1, developed beyond the edges of the paper with (1) semi-stench (60 h) and (2) *n*-butanol–propionic acid–water (20 h). Abbreviations as for Fig. 1.

relative R_F values under over-running conditions with ıeference to 3-phosphoglyceric acid, and a ıadioautogram is shown in Fig. 3. These different mobilities after prolonged chromatographic development may probably be ascribed to three factors: (i) greater esterification in the solvent at increasing times after mixing: this would be

TABLE II

EFFECT OF OVER-RUNNING ON $R_{3\text{-phosphoglyceric acid}}$ OF PHOSPHATES

Chromatograms developed either to edge of paper (12 h for semi-stench; 8 h for *n*-butanol–propionic acid–water) or over-run for 60 h in semi-stench and 20 h in *n*-butanol–propionic acid–water (BuPr).

Compound	$R_{3\text{-phosphoglyceric acid}}$			
	Chromatogram developed to edge of paper only		*Chromatogram over-run*	
	Semi-stench	*BuPr*	*Semi-stench*	*BuPr*
Fructose-6-phosphate	1.03	0.75	0.91	0.73
Fructose diphosphate	0.27	0.29	0.23	0.23
Glucose-6-phosphate	0.77	0.68	0.63	0.66
Phosphoenolpyruvic acid	1.33	1.36	1.53	1.38
2-Phosphoglyceric acid	1.00	1.00	1.24	1.02
3-Phosphoglyceric acid	1.00	1.00	1.00	1.00
Ribose-5-phosphate	1.28	0.88	1.13	0.79
Ribulose diphosphate	0.41	0.46	0.35	0.33
Uridinediphosphoglucose	0.83	0.37	0.74	0.35
Aspartic acid	1.88	1.17	2.22	1.27

TABLE III

ALTERATION OF SOLVENT COMPOSITION DURING CHROMATOGRAM DEVELOPMENT

Percentage (v/v) of each component in the mixture

	Water	*Isopropanol*	*n-Propanol*	*n-Butanol*	*Isobutyric acid*
1. Semi-stench					
Stock bottle	26.5	1.9	9.1	1.9	60.6
Chromatography trough, after development	26.2	1.8	8.5	3.2	60.4
Chromatogram, 0–7.5 cm	18.4	1.0	6.3	7.7	66.5
Chromatogram, 7.5–15 cm	16.6	1.1	6.0	7.7	68.7
Chromatogram, 15–22.5 cm	15.5	1.2	6.3	7.5	69.6
Chromatogram, 22.5–30 cm	16.5	1.2	6.6	7.4	68.3
Chromatogram, 30–37.5 cm	16.0	1.4	6.6	7.5	68.4

	Water	*n-Butanol*	*Propionic acid*
2. n-Butanol–propionic acid–water			
Stock bottle, fresh	30.7	46.8	22.5
Stock bottle, after 24 h	31.5	49.0	19.5
Chromatography trough, after development (24 h old)	25.7	50.1	24.2
Chromatogram, 0–7.5 cm	22.1	50.4	27.6
Chromatogram, 7.5–15 cm	14.9	55.4	29.6
Chromatogram, 15–22.5 cm	15.7	56.1	28.2
Chromatogram, 22.5–30 cm	16.3	57.5	26.2
Chromatogram, 30–37.5 cm	12.9	58.1	29.0

of greater significance with butanol-propionic acid–water, as this solvent is freshly prepared and esterification would thus be progressive; (ii) differential evaporation of component substances in the mixture; (iii) paper chromatographic separation of the solvent constituents themselves, resulting in a series of bands of varying composition along the chromatogram.

Differences in solvent composition at varying stages after preparation and during one-dimensional chromatograms are shown in Table III. After development the wet chromatogram was cut into strips, 7.5 cm wide, perpendicular to the direction of solvent travel. Liquid was obtained from each strip, as well as from residual solvent in the trough. All these samples were analyzed by vapour phase chromatography, using a polymetaphenyl ether column at 90° flushed with helium. Quantitative determinations of the separated components were made by measurements of the peak heights. Small amounts of esters would probably have been obscured by the carboxylic acid peak. It will be seen from Table III that *n*-butanol–propionic acid–water, but not semi-stench, loses water simply by standing in the trough. Both solvents show changes as they flow down the paper, becoming relatively poor in water with increasing distance travelled. Semi-stench also becomes relatively poorer in *n*-propanol and isopropanol and both solvents become richer in *n*-butanol and in the acid component.

Some substances, notably carboxylic acids, have different mobilities in butanol–propionic acid, depending on whether the solvent in the first dimension was semi-stench or phenol–water. This might be explained as a result of the formation of the ammonium salts after development in semi-stench, the ammonium salts then having lower mobilities than the free acids in butanol–propionic acid. This would be particularly significant for the dicarboxylic and oxocarboxylic acids, all of which are considerably stronger acids than propionic.

Two further properties of semi-stench are worthy of mention. Firstly, the flexibility of the system enables its hydrophilicity to be increased or decreased at will to suit particular circumstances. Increasing the water content at the expense of the alcohols allows the more water-soluble components to migrate further, and *vice versa*. The second property is of value in preparing substances for use in microbiological assay and in enzymology. No matter for how long a paper chromatogram is dried it is almost impossible to remove all traces of many solvents, even after development with another solvent. Trace amounts of phenol can poison both enzymic and microbiological systems, while traces of isobutyric acid pose no apparent difficulty in this respect.

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SUMMARY

A new solvent system, to avoid the use of phenol–water, has been developed for the chromatography of complex mixtures of intermediary metabolites. The solvent contains ethylenediaminetetraacetic acid, ammonia, water, isobutyric acid and four simple aliphatic alcohols, and is stable at least for many weeks. The advantages and properties of this solvent are discussed, and the mobilities in it of about one hundred metabolic intermediates are listed with reference to the mobility of aspartic acid.

Note added in proof

It has been found that the chromatographic resolution and the sharpness of the spots are greatly improved by the use of papers other than Whatman No. 4. Whatman No. 2 is distinctly superior to Whatman No. 4, but the best results have been obtained with Ederol No. 202 (J. C. Binzer, Vertriebs-G.m.b.H., Hatzfeld/Eder, West Germany). With these papers the solvent development times are about 30 h for semi-stench and 18 h for *n*-butanol–propionic acid–water.

REFERENCES

[1] A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, *J. Am. Chem. Soc.*, 72 (1950) 1710.
[2] M. Mizell and S. B. Simpson, *J. Chromatog.*, 5 (1961) 157.
[3] A. K. Huggins and V. Moses, *Nature*, 191 (1961) 668.
[4] V. Moses, *J. Chromatog.*, 9 (1962) 241.
[5] R. E. Kay, D. C. Harris and C. Entenman, *Arch. Biochem. Biophys.*, 63 (1956) 14.
[6] V. M. Brenner and A. Niederwieser, *Experientia*, 16 (1960) 378.
[7] E. Tyszkiewicz, *Anal. Biochem.*, 3 (1962) 164.
[8] G. J. Crowley, *Invertebrate Serological Responses, Dissertation*, University of San Francisco, 1962.
[9] J. Ullrich, *University of California Lawrence Radiation Laboratory Quart. Rep.*, UCRL-9900, Sept. 1961, p. 30.

ELECTROPHORETIC METHOD FOR OBTAINING NUCLEOTIDES LABELLED WITH RADIOACTIVE CARBON

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INTRODUCTION

Ion-exchange chromatography is the method most frequently used for quantitative analysis of a mixture of nucleotides.

This method, introduced by COHN[1] in 1950, has been modified by numerous authors[2-6]. Recently NILSSON AND SJUNNESSON[7] described a method of separating nucleotides by means of chromatography on Ecteola-cellulose.

The analysis of a nucleotide mixture can also be carried out successfully by electrophoresis. This method, introduced by MARKHAM AND SMITH[8], makes it possible to obtain good separation of the four mononucleotides occurring in ribonucleic acid (RNA). TURBA, PELZER AND SCHUSTER[9] described a method of separating nucleotides by means of high-voltage electrophoresis; KLOUWEN[10] applied it to the separation of nucleotides extracted from tissues. Column electrophoresis[11] has also been employed.

All the methods mentioned above are used mainly for quantitative analysis of small amounts of nucleotide mixtures. The purpose of our present research was to develop a simple method of obtaining the four mononucleotides of RNA biosynthetically labelled with radioactive carbon.

EXPERIMENTAL

The four mononucleotides were separated by means of continuous electrophoresis on filter paper[12-14]. Electrophoresis was conducted on Whatman 3MM filter paper in an acid buffer solution at pH 3.5 (lithium acetate–acetic acid)[15]; the potential was 270 V, distributed over the whole sheet. The nucleotide mixture was applied continuously to the filter paper by means of a suitable device. The progress of the separation was checked with an ultraviolet lamp (Mineralight). The concentration of the nucleotides in the different test tubes was determined spectrophotometrically (a spectrophotometer of the Hilger type) in acid medium (pH 2.0) in 1 cm quartz cells.

Verification of the method

A sample of pure yeast RNA (British Drug Houses, Ltd., London) hydrolysed by the method of SCHMIDT AND TANNHAUSER[16] was used to determine the optimum con-

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ditions for the separation of the four constituent mononucleotides. The RNA hydrolysate, acidified to pH 5.0, was introduced on to a column of suitably prepared activated charcoal[17]. The nucleotides were then eluted from the column with a solution of ammonia in alcohol. After evaporation to dryness, the eluate was transferred in 1 ml 0.1 *N* HCl to the dispensing vessel, and continuous electrophoresis carried out. The separation of the individual nucleotides on filter paper is shown in Fig. 1.

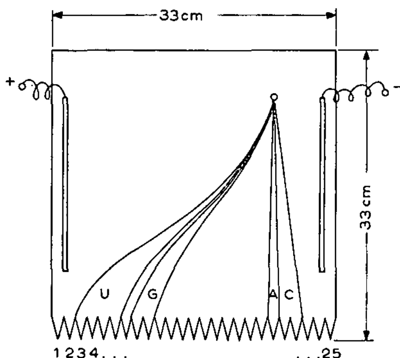

Fig. 1. Diagram of the electropherogram of the four mononucleotides. A = AMP; C = CMP; G = GMP; U = UMP.

The fractions collected in the test tubes were acidified to pH 2.0 and the absorption was determined for each of them at the following four wave lengths 250, 260, 275 and 280 mμ. The ratios of the absorption coefficients thus obtained are given in Table I, and were compared with those obtained by COHN[18] for pure nucleotides.

As can be seen from Table I, the absorption coefficient ratios calculated for the nucleotides in the present experiment differed slightly from those calculated for pure nucleotides. It was found that the buffer used in our experiments had a certain

TABLE I

ABSORPTION COEFFICIENTS OF THE FOUR MONONUCLEOTIDES

Nucleotide		*250/260*	*275/260*	*280/260*
AMP	T	0.85	0.43	0.22
	E	0.92	0.43	0.25
CMP	T	0.45	2.00	2.10
	E	0.52	1.65	1.67
GMP	T	0.96	0.69	0.67
	E	0.97	0.73	0.70
UMP	T	0.74	0.64	0.38
	E	0.86	0.59	0.36

T = theoretical values; E = experimental values.

TABLE II

ABSORPTION OF FILTER PAPER BACKGROUND

λ	250 $m\mu$	260 $m\mu$	275 $m\mu$	280 $m\mu$
$E \times 100/1$ ml	41.3	34.2	20.2	17.8

degree of absorption in the ultraviolet part of the spectrum. The absorption values due to the filter paper alone were determined for 1 ml of eluate at the four wave lengths and are listed in Table II.

Some absorption was found in the eluate from the filter paper alone, the greatest absorption being in the region of 250 $m\mu$. This could account for the slight deviation of our absorption coefficients from the theoretical values.

For quantitative determination of the individual nucleotides we used the extinction value obtained at 260 $m\mu$ less absorption of the background at this same wave length. Alternatively the quantity of adenosine monophosphate (AMP), guanosine monophosphate (GMP) and uridine monophosphate (UMP) can be determined from the linear relationship between the difference in absorption at 260 and 280 $m\mu$ and concentration; this artifice eliminates the effect of the background absorption.

The value calculated in this way for cytidine monophosphate (CMP) was lower than the actual content of the nucleotide. We also determined the absorption curves of the individual nucleotides using the formula for the atomic extinction coefficient[19].

$$\varepsilon(P) = \frac{30.98 \times E}{c \times 1}.$$

The absorption curves obtained in the present experiment are shown in Fig. 2, where it can be seen that the characteristics of the curves are similar to those ob-

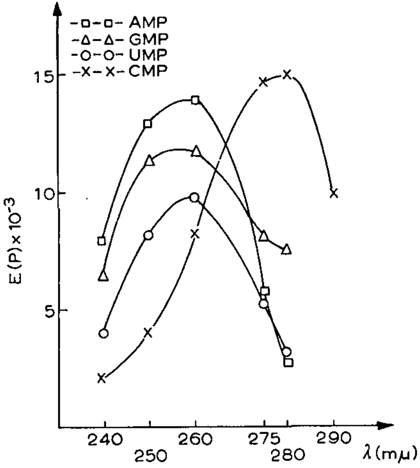

Fig. 2. Absorption curves of the four nucleotides separated by continuous electrophoresis. □——□ AMP; ×——× CMP; △——△ GMP; ○——○ UMP.

tained for pure nucleotides. This confirms the efficacy of the separation and the purity of the nucleotides so obtained.

In view of the practical character of the present method, it seemed expedient to determine the losses resulting from the technical operations, or connected with the degree of separation of the different nucleotides on the filter paper. After applying 1.0 mg of nucleotide-P to the filter paper by the continuous method, 60 to 75 % of nucleotide-P can be obtained in the form of pure nucleotides. The extent of loss depends, apart from other factors, on the degree of separation of the bands of AMP and CMP. It can be seen from Fig. 1, that AMP and CMP occur close together on the filter paper, so that a "tongue" of the filter paper sometimes furnishes a mixture of both nucleotides.

With the present method, provided that a suitable concentration of the mixture is applied to the filter paper, 6 to 10 μmoles of each mononucleotide can be obtained after 18 h electrophoresis.

Separation of nucleotides biosynthetically labelled with ^{14}C

Having established the general principles of the method for separation we proceeded to prepare ^{14}C-labelled nucleotides by biosynthesis. The method used was based on that of DOWNING AND SCHWEIGERT[20] AND MANTSAVINOS AND CANELLAKIS[21] for the production of desoxyribonucleotides. In our experiments *E. coli* was used, which when incubated in the presence of $NaH^{14}CO_3$, incorporates ^{14}C into the nucleic acids. The initial radioactive compound was $Ca^{14}CO_3$, which was converted to sodium bicarbonate in the Van Slyke–Folch apparatus.

500 μC of $Ca^{14}CO_3$ was used for a single experiment. The $NaH^{14}CO_3$ was added to the incubation medium, simultaneously with a suspension of *E. coli*, which had been passed through a synthetic medium in the presence of non-radioactive sodium bicarbonate. The synthetic incubation medium used in the labelling process contained glucose, sodium citrate and radioactive sodium bicarbonate as sources of carbon. Incubation was carried on for 18 h at 37° in an apparatus which prevented radioactive carbon dioxide from escaping into the atmosphere. Under these conditions intense multiplication of the bacterial cells takes place, and radioactive carbon is incorporated into the cellular components. Growth was stopped by heating the bacteria suspension to 50°, and the culture was aerated at the same temperature in order to remove the remaining carbon dioxide from the medium.

The bacterial mass was centrifuged, washed twice with cold 0.9 % NaCl, and then lyophilized. The dry powder was extracted twice with anhydrous ethyl ether at 0°, followed by two extractions with 0.6 *M* $HClO_4$ at 0°, and then washed with water. The sediment was extracted twice with boiling ethanol, and twice with a mixture of methanol and chloroform. The powder was then subjected to alkaline hydrolysis. The nucleoside-3′-monophosphates so obtained were purified on a column of activated charcoal and then separated by means of continuous electrophoresis according to the method already described.

The radioactivity of the different fractions was determined, during the successive stages of the experiment. This was done by counting a dried layer of the sample with a Geiger-Müller counter having a mica window (1.4 mg/cm^2); the counter was placed 10 mm from the sample. The concentration of RNA was determined by means of a spectrophotometer, the calculations being carried with out the coefficients given by

TSANIEV[22]. The distribution of the isotope label over the different stages of the experiment is shown in Table III where it can be seen that the radioactivity of the non-purified RNA preparation is much higher than that obtained after purification on a charcoal column. The radioactivity of each of the isolated mononucleotides approaches that of RNA, the isotope label being distributed evenly over all the four mononucleotides.

TABLE III

DISTRIBUTION OF RADIOTRACER IN THE RADIOACTIVE NUCLEOTIDES OBTAINED BY BIOSYNTHESIS

Analysed compound	*Quantity of analysed compound in μg P*	*Activity C/min/μg P*	*Specific activity C/min/μmole*
RNA non purified	828.0	975	—
RNA after purification	746.0	570	—
AMP	101.5	510	15,800
CMP	107.4	510	15,800
UMP	172.2	516	16,000
GMP	168.6	480	14,600
Adenine	45.0 μg	—	14,700
Cytosine	63.0 μg	—	14,300
Uracil	52.0 μg	—	15,200
Guanine	41.8 μg	—	14,300

In order to discover the position of the radioactive carbon in the nucleotides obtained in the present experiment the pure preparations were hydrolysed. Purine nucleotides were hydrolysed in 1 *N* HCl for 1 h at 100°.

After vacuum drying at a low temperature, the hydrolysate residue was dissolved in water and applied to the filter paper. Pyrimidine nucleotides were hydrolysed in 60 %: $HClO_4$ for 1 h at 100°. The perchloric acid was removed by cold precipitation in the form of $KClO_4$. The pure hydrolysate was condensed by lyophilization, and then dissolved in a small quantity of water, and applied to the filter paper.

Descending chromatography was carried out in the isopropanol–HCl[23] or in butanol–ammonia[8] for 24 h. The ribose spots on the chromatogram were localized by the aniline test[24], or by the silver nitrate test[25]. Spots of purine and pyrimidine bases were observed in ultraviolet light, a suitable filter being used in the lamp. In addition, the radioactivity was determined by direct measurement on the chromatograms.

The distribution of the isotope on the chromatograms is shown in Fig. 3. The whole of the activity was found to be in the purine base, whereas the activity in the spot where ribose is located on the chromatogram was equal to that of the background. The purine and pyrimidine bases separated chromatographically from the radioactive nucleotides were eluted from the chromatograms with 0.1 *N* HCl.

The concentration of the individual bases in the eluates was determined spectrophotometrically, and their activity measured. The specific activities found for the individual bases are listed in Table III; they hardly differ from those of the nucleotides from which the bases had been obtained. It can be concluded that the radioactive carbon is only incorporated into the base part of the nucleotide.

In the experiments in which 500 μC of sodium bicarbonate was added to every 700 ml of the incubation medium, the isolated nucleotides differed only slightly

from one another in their specific activity. In one experiment, in which 600 μC $NaH^{14}CO_3$ was used, the activity of the obtained nucleotides was proportionally higher.

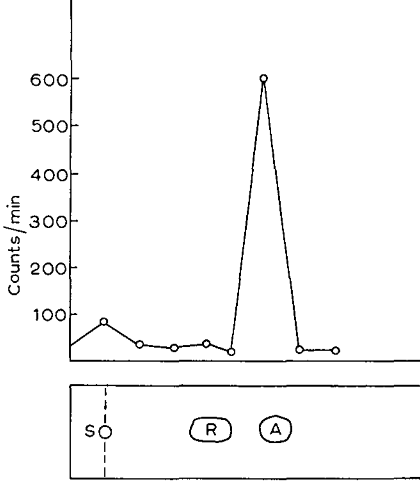

Fig. 3. Distribution of the radiotracer in the hydrolysate of adenylic acid as revealed by paper chromatography. S = starting point; R = ribose; A = adenine.

DISCUSSION

Unlike most methods mentioned in the introduction, our method of separating the four mononucleotides is characterized by great simplicity. MANTSAVINOS AND CANELLAKIS, using chromatography on a column with Dowex 1, obtained from 400 mg of lipid-free bacteria powder, 8 to 13 μmoles of each of the desoxynucleotides of DNA. Our method also makes it possible to obtain 6 to 10 μmoles of each ribonucleotide. Detailed analysis demonstrated that the nucleotides obtained by our method were of high purity and the losses resulting from all operations connected with the electrophoresis did not exceed 30 %.

The analysis of the activity of the individual fractions obtained in our studies on ^{14}C-labelled nucleotides confirmed the fact that ^{14}C is incorporated into various chemical compounds. Both the fraction of acid-soluble compounds and the lipid fraction of the culture of *E. coli* showed a high degree of activity. An equally high activity was found in a neutralized alkaline hydrolysate of bacterial RNA. After purification of the hydrolysate on a charcoal column, the activity per μg of nucleotide-P decreased considerably in comparison with the corresponding value in a non-purified alkaline hydrolysate. On the other hand, the activity of the individual nucleotides did not differ from the activity of the sum of nucleotides contained in the RNA purified on a charcoal column. It is known from the work of TSANIEV AND MARKOV[20], and FLECK AND MUNRO[26] that when biological material is subjected to alkaline hydrolysis, not only nucleotides, but also products of protein decomposition, such as peptides and phosphopeptides pass into the hydrolysate. A comparison of the values, obtained in the present research, for the activity of RNA before and after purification indicates the presence in the alkaline hydrolysate of non-

nucleotide products of high activity. These findings might confirm the observation that in the case of bacteria, $^{14}CO_2$ is also incorporated into amino acids. It was of more interest to us to investigate to what degree the bases and the ribose in the nucleotides had been labelled.

Our experiments demonstrated that the whole of the radiotracer is located in the purine and pyrimidine bases as the bases obtained from nucleotides showed the same specific activity as the nucleotides themselves.

In the majority of studies carried out with radioactive carbon, a flow counter is used to measure the activity as it is a better detection of soft β-rays; however, a Geiger-Müller counter with a mica window was used in the present experiments. Thus the specific activity found by us for the individual nucleotides represents a high degree of incorporation of isotope, as the efficiency of the counter is low for ^{14}C It would seem that the ^{14}C-labelled nucleotides obtained in our experiments could be used for investigating the synthesis of RNA.

SUMMARY

The conditions for separating AMP, GMP, CMP and UMP by means of continuous electrophoresis on Whatman 3 MM filter paper in acid buffer at pH 3.5 are described. The ribunucleotides of RNA obtained in this way were tested with regard to their purity.

The method can be used for the production of the four mononucleotides on a laboratory scale. Good results were obtained for the production of nucleotides from biosynthetically labelled RNA of *E. coli* grown on a synthetic medium in the presence of $^{14}CO_2$.

The radioactive mononucleotides were found to be labelled in the purine and pyrimidine bases only. The specific activity of the four mononucleotides was similar.

REFERENCES

[1] W. E. COHN, *J. Am. Chem. Soc.*, 72 (1950) 1471.
[2] W. E. COHN, *J. Biol. Chem.*, 203 (1953) 319.
[3] R. BERGKVIST AND A. DEUTSCH, *Acta Chem. Scand.*, 8 (1954) 1877.
[4] R. B. HULBERT, H. SCHMITZ, A. F. BRUMEN AND V. R. POTTER, *J. Biol. Chem.*, 209 (1954) 23.
[5] H. G. PONTIS AND N. L. BLUMSON, *Biochim. Biophys. Acta*, 27 (1958) 618.
[6] R. K. CRANE AND F. LIPMANN, *J. Biol. Chem.*, 201 (1953) 235.
[7] R. NILSSON AND M. SJUNNESSON, *Acta Chem. Scand.*, 15 (1961) 1017.
[8] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.
[9] F. TURBA, H. PELZER AND H. SCHUSTER, *Z. Physiol. Chem.*, 296 (1954) 97.
[10] H. M. KLOUWEN, *J. Chromatog.*, 7 (1962) 316.
[11] N. FRONTALI, *Acta Chem. Scand.*, 13 (1959) 390.
[12] H. SVENSON AND L. BRATSON, *Arkiv Kemi, Mineral. Geol.*, 1 (1949) 401.
[13] W. GRASSMAN AND K. HANNIG, *Naturwiss.*, 37 (1950) 397.
[14] W. GRASSMAN AND K. HANNIG, *Z. Physiol. Chem.*, 292 (1953) 32.
[15] N. VIRMEAUX, personal communication.
[16] G. SCHMIDT AND S. J. TANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
[17] J. R. BERTLETT, *J. Biol. Chem.*, 234 (1959) 465.
[18] W. E. COHN, in S. P. COLOWICK AND N. O. KAPLAN (Editors), *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 724.
[19] E. CHARGAFF AND S. ZAMENHOF, *J. Biol. Chem.*, 173 (1948) 327.
[20] M. DOWNING AND B. S. SCHWEIGERT, *J. Biol. Chem.*, 220 (1956) 513.
[21] R. MANTSAVINOS AND E. S. CANELLAKIS, *J. Biol. Chem.*, 234 (1959) 628.
[22] R. TSANIEV AND G. G. MARKOV, *Biochim. Biophys. Acta*, 42 (1960) 442.
[23] G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
[24] S. M. PARTRIDGE, *Nature*, 164 (1949) 443.
[25] W. Z. TRAVELYAN, O. D. PROCTOR AND J. S. HARRISON, *Nature*, 166, (1950) 444.
[26] A. FLECK AND H. N. MUNRO, *Biochim. Biophys. Acta*, 55 (1962) 571.

ANION EXCHANGE SEPARATIONS OF ALDOBIONIC AND ALDONIC ACIDS

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An anion exchange method for the separation of aldonic acids in borate medium described in earlier papers[1,2], has been applied to the analysis of sulfite spent liquors[3,4] and to the determination of aldonic acid end groups in cellulose[5] and hemicellulose[6]. In connection with this work it was found desirable to develop methods which permit separations in systems involving aldobionic acids. The present work deals with the separation of aldobionic acids and aldonic acids both in borate and acetate media.

EXPERIMENTAL

Preparation of barium aldobionates

Cellobionic, maltobionic, lactobionic and melibionic acids have been prepared by electrolyte oxidation of the corresponding disaccharides. The conditions were, with a few exceptions, the same as those described by ISBELL AND FRUSH[7]. The reaction mixture consisted of 0.1 mol disaccharide, 10 g $CaCO_3$, and 4 g $CaBr_2 \cdot 2H_2O$ in 400 ml water. The distance between the graphite anode and the platinum cathode was kept as small as possible in order to obtain a low potential (3-4 V). The reaction was carried out in the dark[8] and was allowed to proceed until 85–90 % of the sugar had been oxidized.

After the oxidation step, nitrogen gas was bubbled through the solution. A slight excess of silver carbonate was added and the solution was stirred in the dark for a few minutes. After filtration, the solution was tested for bromide. The solution was then cooled with ice and stirred with 100 ml of a cation exchanger in its free-acid form (Dowex 50 X-4; 50–100 mesh) for 2 min. After filtration, the acid solution was brought in contact with 100 ml of a strongly basic anion exchange resin in its bicarbonate form (Dowex 1 X-2; 60–140 mesh). After stirring for 15 min, the resin was transferred to a column and washed with water until the anthrone test was negative. Nitrogen pressure was applied to increase the flow rate. The column was emptied and the resin stirred with 100 ml of ice-cooled 0.5 *M* sulfuric acid to remove carbon dioxide. The acid washed resin was then transferred to a column and the elution of aldobionic acid was completed by treating with 150 ml of the cold acid and washing with 500 ml water. The time of elution was 10–15 min; the washing required about 30 min. The eluate and washings were collected in a beaker containing barium carbonate. The solution was heated to boiling and the precipitate removed by centrifugation. The solution was evaporated under vacuum (30°) to a small

volume and added to a tenfold volume of absolute ethanol in a mortar. The barium salt appeared as a white powder which was separated from the solution by centrifugation, washed with ethanol, and dried between filter papers over phosphorus pentoxide. The yield was 70–75%.

Chromatographic procedures

The separations in acetate medium were carried out with a strongly basic anion exchange resin in the acetate form (Dowex 1 X-8; 40–60 μ). The column, which had a diameter of 10 mm and a length of 920 mm, was loaded with a solution of barium salts in about 5 ml of water. After washing with water, elution was carried out at a flow rate of 1.3 $ml.cm^{-2}.min^{-1}$. The temperature was kept at 25°. The standard technique with a motor-driven pump for feeding the eluant onto the column and a time-actuated fraction collector were employed[9]. The eluate fractions were analyzed by chromic acid oxidation using the Technicon AutoAnalyzer[3].

The experiments with the borate resin were carried out analogously. If not otherwise mentioned, the column dimensions were 10 × 1460 mm. The temperature was 30°. Under the elution conditions used in this work, the peak elution volumes are higher in borate medium. This results in broader elution bands, and in order to make the analysis of the eluate as simple as possible larger amounts of the acids were used in these experiments than in the separations in acetate medium.

RESULTS AND DISCUSSION

Separations in acetate medium

In an earlier paper it was shown that several hydroxy acids can be easily separated by elution with sodium acetate solution[10]. Acids with a higher molecular weight, such as glucosaccharinic acid, appeared ahead of those with a lower molecular weight, such as lactic and glycolic acids. It could, therefore, be expected that at the same

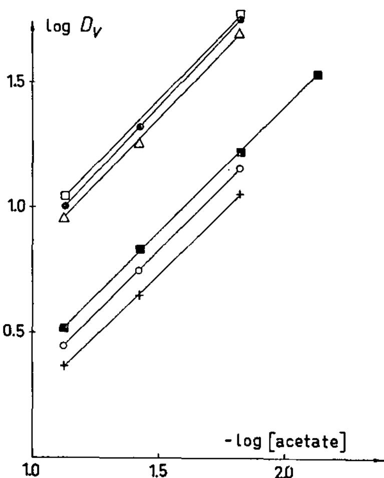

Fig. 1. Influence of the sodium acetate concentration upon the volume distribution coefficient, D_V. □ rhamnonic acid; ● mannonic acid; △ xylonic acid; ■ cellobionic and maltobionic acids; ○ lactobionic acid; + melibionic acid.

eluant concentration the aldobionic acids would appear before any of the acids studied in the previous investigation. This was confirmed experimentally. The peak elution volumes have been determined at various eluant concentrations and the volume distribution coefficients (D_v) calculated as usual[9]. In Fig. 1 log D_v has been plotted against –log [acetate]. Straight lines were obtained with a slope of $+1$ (*cf.* ref. 9), which is in agreement with theory. There were significant differences between the distribution coefficients of melibionic, lactobionic and cellobionic acids, whereas the difference between cellobionic and maltobionic acid is, over the whole range of concentration, within the limits of experimental error. Sodium acetate is unsuitable as an eluant for the separation of cellobionic and maltobionic acids. An experiment with both acids on the same column showed that only one elution peak was obtained even at a very low eluant concentration (0.0075 M).

The elution behavior of some simple aldonic acids in acetate medium is also demonstrated in Fig. 1. It is seen that the aldonic acids are held much more strongly by the resin than the aldobionic acids, which is in agreement with the previously mentioned observation that many hydroxy acids appear in the order of decreasing molecular weight. This behavior may serve as a preliminary indication when identifying unknown mixtures, but xylonic acid, which contains one carbon atom less than mannonic acid, is held less firmly, which shows that this rule is not always valid.

From the experimental data it can be concluded that a group separation of aldonic acids from aldobionic acids can be carried out very easily in acetate medium. In the experiment presented in Fig. 2 cellobionic acid is separated quantitatively from mannonic acid in 0.075 M sodium acetate solution. At this eluant concentration the mutual separation of various acids within each group is far from complete.

Although the separation factor, *i.e.*, the ratio between the distribution coefficients for the various aldobionic acids depends only slightly upon the eluant concentration (Fig. 1), their mutual separation is improved to a large extent when the concentration is lowered. This improvement, according to Glueckauf's theory (*cf.* ref. 9), can be explained by the increased number of theoretical plates at a lower concentration. Similar results have been reported in an earlier paper for the separation of lactic and glycolic acids[10] in acetate medium. A chromatogram of a mixture

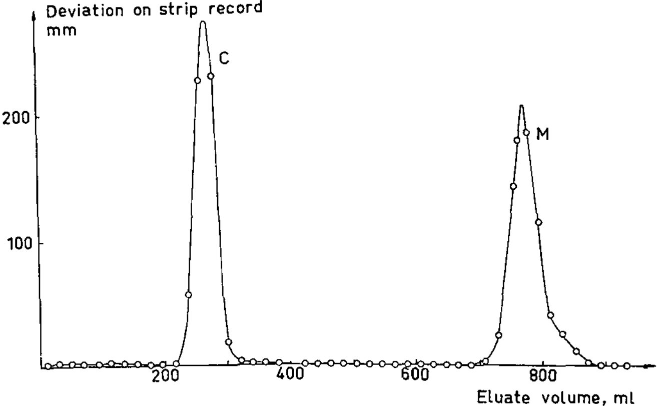

Fig. 2. Separation of 20 mg cellobionic acid (C) from 19 mg mannonic acid (M). Eluant: 0.075 M sodium acetate.

of melibionic, lactobionic, and cellobionic acids together with gluconic and mannonic acids run in 0.015 M sodium acetate is reproduced in Fig. 3, where it can be seen that the separation of the aldobionic acids from each other and from the aldonic acids is satisfactory for analytical purposes. The two aldonic acids are also separated, but at

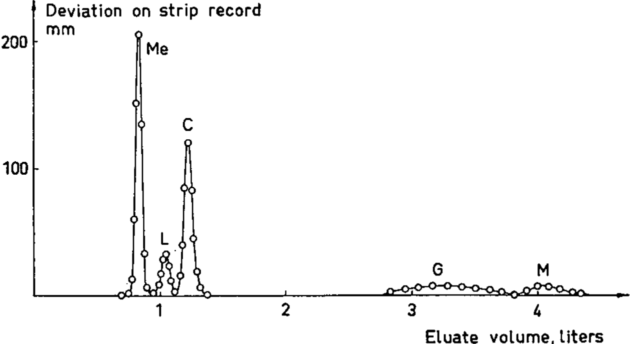

Fig. 3. Separation of various aldobionic and aldonic acids. Eluant: 0.015 M sodium acetate. Me: melibionic acid (17 mg); L: lactobionic acid (8 mg); C: cellobionic acid (19 mg); G: gluconic acid (17 mg); M: mannonic acid (10 mg).

this low eluant concentration the elution curves are wide and less suitable for quantitative calculations. In systems where several aldobionic acids are present together with only one simple aldonic acid it is preferable to use a stepwise elution, *i.e.*, to increase the eluant concentration after the aldobionic acids have been eluted.

Fig. 4 shows that xylonic and mannonic acids can be almost completely separated even at a higher sodium acetate concentration. However, the separation factors for the simple aldonic acids are, in general, less favorable in acetate medium than in borate medium.

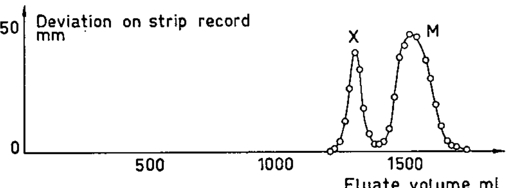

Fig. 4. Separation of 10 mg xylonic acid (X) from 20 mg mannonic acid (M). Eluant: 0.0375 M sodium acetate.

Separations in borate medium

In borate medium the aldonic acids appear in the following order: xylonic, arabonic, mannonic, gluconic, galactonic acid[1]. The pentonic acids appear ahead of the hexonic acids. The uptake can be ascribed to the combination of ion exchange due to the presence of a carboxyl group and the formation of borate complexes in which the hydroxy groups are involved. Rhamnonic acid (6-deoxy-mannonic acid), which contains one hydroxy group less than the hexonic acids, would be expected to appear before the hexonic acids. As can be seen in Fig. 5, rhamnonic acid appears even before xylonic acid, *i.e.*, it is first among all the aldonic acids studied. A qualitative separation can thus be obtained of all aldonic acids hitherto investigated. A comparison

between the results obtained in borate medium with those in acetate medium shows that borate solutions are preferable when a complicated mixture of aldonic acids is to be separated.

Certain systems containing a mixture of aldonic and aldobionic acids can be satisfactorily analyzed by the borate method. The separation of rhamnonic, xylonic,

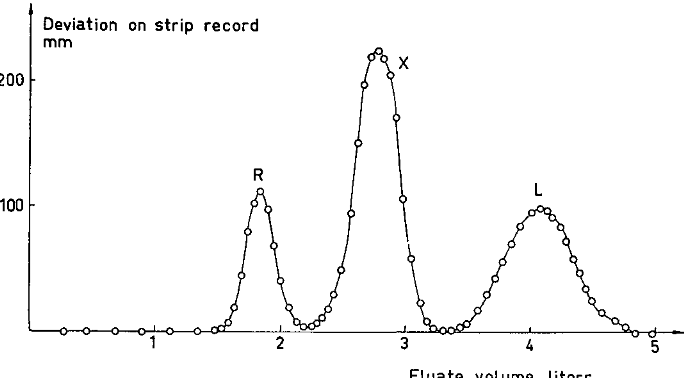

Fig. 5. Separation of 70 mg rhamnonic acid (R), 140 mg xylonic acid (X), and 150 mg lactobionic acid (L). Eluant: 0.1 M sodium tetraborate.

and lactobionic acids is indicated in Fig. 5. Similarly, rhamnonic acid can be separated from melibionic acid (Fig. 6). Cellobionic acid is also eluted as a well separated band after rhamnonic acid, but the peak elution volume differs too little from that of melibionic acid to permit a quantitative separation at the eluant concentration used in these experiments (0.1 M). Working at a lower concentration is known to give an improved separation of other acids, but the elution is tedious and the elution bands are broadened to such an extent that the accuracy is decreased in quantitative determinations.

It can be concluded from the experimental results that the acetate method is preferable when melibionic, lactobionic, and cellobionic (or maltobionic) acids are to be separated from each other, but the acetate method fails to separate cellobionic and maltobionic acids. The separation of cellobionic acid from maltobionic acid is

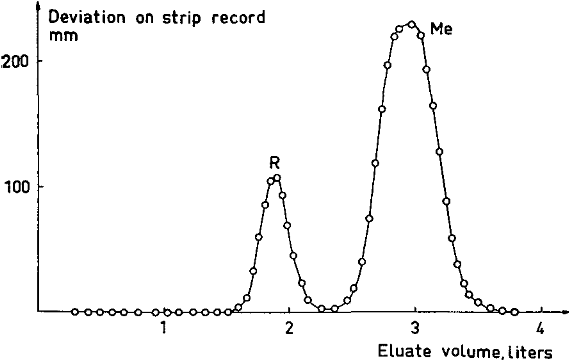

Fig. 6. Separation of 70 mg rhamnonic acid (R) from 160 mg melibionic acid (Me). Eluant: 0.1 M sodium tetraborate.

very difficult in borate medium. Using two columns coupled in series it is possible to achieve a partial separation in 0.1 *M* sodium tetraborate solution (Fig. 7). The curves overlap to such an extent that the method can be used only for semi-quantitative determinations of these acids.

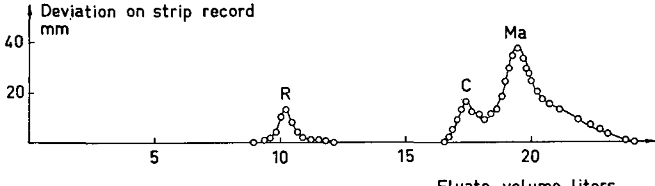

Fig. 7. Separation of 70 mg rhamnonic acid (R), 70 mg cellobionic acid (C) and 180 mg maltobionic acid (Ma). First column: 20 × 1480 mm. Second column: 10 × 920 mm. Flow rate: 2 ml/min. Eluant: 0.1 *M* sodium tetraborate.

In borate medium the aldobionic acids studied have peak elution volumes which differ only slightly from those of the aldonic acids which appear after rhamnonic acid. For this reason the borate method is unsuitable when complicated mixtures of aldonic acids are to be separated from aldobionic acids. When such mixtures are to be analyzed, a group separation is first carried out in acetate medium. If desired, melibionic, lactobionic, and cellobionic acids (together with maltobionic acid), which appear first in the eluate (Fig. 3), can be separated as three elution bands. After cellobionic acid has been eluted the acetate concentration is increased to 0.1 *M* and the aldonic acids are eluted as a group. Subsequently, the borate method is employed to separate all aldonic acids as individual bands.

ACKNOWLEDGEMENT

The financial support of the Swedish Technical Research Council is gratefully acknowledged.

SUMMARY

Several aldobionic acids can be separated from each other by chromatography on anion exchange resins. These separations as well as mutual separations of some simple aldonic acids can be carried out by elution with sodium acetate. When complicated mixtures of aldonic acids are to be separated from aldobionic acids, the aldobionic acids are separated in acetate medium. The aldonic acids are eluted as a group and, subsequently, separated in borate medium.

REFERENCES

1 O. Samuelson, K. J. Ljungqvist and C. Parck, *Svensk Papperstidn.*, 61 (1958) 1043.
2 O. Samuelson and R. Simonson, *Svensk Papperstidn.*, 65 (1962) 363.
3 O. Samuelson and R. Simonson, *Svensk Papperstidn.*, 65 (1962) 685.
4 S.-I. Nord, O. Samuelson and R. Simonson, *Svensk Papperstidn.*, 65 (1962) 767.
5 B. Alfredsson, W. Czerwinsky and O. Samuelson, *Svensk Papperstidn.*, 64 (1961) 812.
6 E. Eriksson and O. Samuelson, *Svensk Papperstidn.*, 66 (1963) 298.
7 H. S. Isbell and H. L. Frush, *Bur. Standards J. Research*, 6 (1931) 1145.
8 C. S. Hudson and H. S. Isbell, *Bur. Standards J. Research*, 3 (1929) 57.
9 O. Samuelson, *Ion Exchange Separations in Analytical Chemistry*, Almqvist & Wiksell, Stockholm, John Wiley & Sons, New York, 1963.
10 B. Alfredsson, S. Bergdahl and O. Samuelson, *Anal. Chim. Acta*, 28 (1963) 371.

Short Communication

Gas chromatography in the analysis of urinary pregnanediol*

Separation of a wide range of pure steroids by gas chromatography has been accomplished recently (reviewed by HORNING *et al.*[1]). Rapidity is a great advantage of this technique over other chromatographic methods used in steroid analysis. Little work has yet been reported on the application of gas chromatography to the analysis of urinary steroids as distinct from pure steroids. In this study the gas chromatographic technique was evaluated as a method which could give rapid and accurate results in the analysis of pregnanediol in pregnancy urine.

Apparatus

The gas chromatograph used was a Pye Argon Model 12001 with a 20 mC ^{90}Sr β-ionization detector. A septum-injector and pre-heater stage were added to the standard equipment. The pre-heater temperature was 250°. Injections of 2 μl of solutions of steroids or extracts in absolute ethanol were made onto 4 ft. long columns, 5 mm internal diameter, packed to a length of approximately 110 cm.

Suitable column packings and liquid phases were selected from the behaviour of pure pregnanediol and other steroids, and re-investigated to determine their applicability to urine extracts. Two selected for work with extracts were (a) the non-polar 3 % "Embaphase" silicone oil, (May & Baker Ltd., London) on Gas Chrom Z, (Applied Science Laboratories) used at 240°, and (b) the polar phase 0 5 % neopentylglycol adipate (NGA) on Gas Chrom Z, 100/140 mesh used at 210-220°.

Chromatography of pure steroids

The behaviour of the columns with pure steroids is shown in Table I.

All these major urinary neutral steroids are well separated from pregnanediol on the adipate column. Amounts of 1–10 μg of pregnanediol were normally applied to the columns. In actual running, pregnanolone and pregnanediol were not completely separated on the 3 % embaphase but were readily separated on 1 % or 0.5 % neopentylglycol adipate columns. The total of pregnanolone and pregnanedione was found to be less than 20 % of the pregnanediol levels in the 50 urines examined. Further results refer to adipate columns.

Chromatography of urine extracts

Extracts were prepared as in the first stage of the procedure of KLOPPER *et al.*[2], using an acid hydrolysis, toluene extraction, alkali partition and permanganate oxidation of impurities (extract B, see below). For gas chromatography, extract residues were dissolved in 50 μl of ethanol. From these solutions, 2 μl aliquots were injected into

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the chromatograph. Solutions in ethanol gave sharper peaks than those in chloroform or tetrahydrofuran.

With pregnancy urine extracts a clear separation of a peak corresponding to pregnanediol has been obtained with no interference from other peaks. Typical results

TABLE I

GAS CHROMATOGRAPHY OF PURE STEROIDS

Steroid	*Retention times relative to cholestane*	
	(a) on 3% NGA at 220°*	*(b) on 0.5% embaphase at 240°*
Cholestane**	1.00	1.00
5β-Pregnane-3α,20α-diol	4.31	0.68
5β-Pregnan-3α-ol-20-one	3.52	0.66
5β-Pregnane-3,20-dione	3.64	0.65
Androsterone	2.39	0.39
Aetiocholanolone	2.39	0.38
Dehydroepiandrosterone	2.64	0.38
11-Oxo-androsterone	5.75	0.48
11-Oxo-aetiocholanolone	5.36	0.44
11-Hydroxy-aetiocholanolone	10.1	0.73

* Neopentylglycol adipate
** *Cholestane retention time* was (a) 2.0 and (b) 7.9 min with the above columns at an outlet argon gas flow rate of 60 ml/min.

are illustrated in Fig. 1. A pregnanediol peak was observed to be one of the main peaks in extracts from 50 urines of the 2nd to 8th month of pregnancy. For late pregnancy urines the pregnanediol peak was the major one observed. For early pregnancy, peaks corresponding to mixtures of androsterone, aetiocholanolone and dehydroepiandrosterone were similar in magnitude to that of pregnanediol but well separated rom it as would be expected from the behaviour of pure steroids (Table I). Six

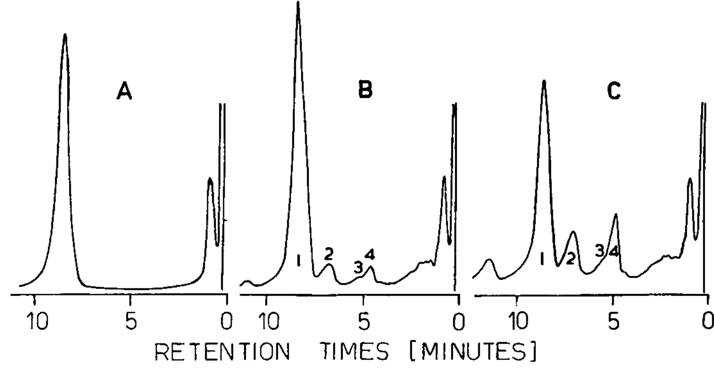

Fig. 1. Gas chromatography on 0.5% NGA at 220°; Argon flow 60 ml/min. Pure pregnanediol 6.3 μg (A); urine extracts from a patient 25 weeks pregnant, pregnanediol 32 mg/24 h (B); and from a patient 16 weeks pregnant, pregnanediol 11 mg/24 h (C). Steroids indicated as present: (1) pregnanediol; (2) pregnanolone and pregnanedione; (3) dehydroepiandrosterone; (4) aetiocholanolone and androsterone.

recovery experiments from urine gave 92–103% recoveries for pregnanediol. For 20 extracts examined as neutral toluene extracts without a permanganate wash, the recording obtained was practically the same as with the wash (extract B).

For non-pregnancy urine, exploratory experiments with 4 urines containing from 0.8–2 mg/24 h. pregnanediol showed that a pregnanediol peak could be observed, but it was a relatively minor one in the presence of other strong peaks, such as those of 17-oxosteroids. For such extracts, good chromatograms with pregnanediol as a major peak were obtained following a prior purification that occurs on an alumina chromatogram as in the KLOPPER procedure. An alternative for non-pregnancy urine may be the formation of the trimethylsilyl derivatives as noted by HORNING *et al.*[1].

Identity of the "pregnanediol" peak

Firstly a comparison was made of the quantitative results by gas chromatography on extracts at stage B and the full sulphuric acid chromogen procedure of KLOPPER *et al.*[2]. For quantitation by gas chromatography the area of peaks was determined by triangulation and compared to standard calibration values run on the same day. Results obtained by the two methods are shown in Table II. Good agreement was obtained between the methods for the pregnancy urines, each of which was from a different subject.

Secondly, portions of the extracts from various later stages in the KLOPPER procedure were taken for gas chromatography to observe the number of other peaks appearing and the resolution of pregnanediol and pregnanediol diacetate from them

TABLE II

QUANTITATIVE ESTIMATION OF URINARY PREGNANEDIOL

Sample	*Pregnanediol found (mg/24 h)*	
	(a) by gas chromatography	*(b) by sulphuric acid chromogens*
Pregnancy urine 1	37	38
Pregnancy urine 2	28	30
Pregnancy urine 3	8.9	9.0
Pregnancy urine 4	7.5	8.3
Pregnancy urine 5	4.2	3.8
Follicular phase urine	0.5	0.8

as extracts were purified. An alumina chromatogram of the urine extracts (stage B) removes a number of relatively strong peaks including those corresponding to androsterone, dehydroepiandrosterone, aetiocholanolone and most of the pregnanolone. The pregnanediol peak was practically the only one present after this alumina purification. It was significant that the other alumina chromatogram fractions gave no peak in the region of pregnanediol on gas chromatography. After acetylation, the only strong peak that was observed, corresponded to pregnanediol diacetate. After further alumina chromatography of the acetylated material, only a single peak was eluted on gas chromatography, with a retention time which corresponded to that of pregnanediol diacetate. This indicates the homogeneity of the pregnanediol peak observed on gas chromatography of extracts at stage B.

Using cyanoethylpolysiloxane on Gas Chrom P, TURNER *et al.*[3] also reported promising results in the estimation of pregnanediol in pregnancy urine. Their conditions did not separate pregnanediol from pregnanolone however. Some preliminary data on urinary pregnanediol separations have been given by PATTI *et al.*[4] with SE 52 columns.

The results presented above indicate that gas chromatography can be used for rapid and accurate analysis of pregnanediol in pregnancy urine, following a very simple hydrolysis and extraction procedure. Further work is in progress to establish fully the specificity, accuracy and reproducibility of the procedure as a standard analytical technique for pregnanediol estimation.

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1 E. C. HORNING, W. J. A. VANDENHEUVEL AND B. G. CREECH, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 11, Interscience, New York, 1963, p. 69.
2 A. KLOPPER, E. MICHIE AND J. B. BROWN, *J. Endocrinol.*, 12 (1955) 209.
3 D. A. TURNER, G. E. S. JONES, I. J. SARLOS, A. C. BARNES AND R. COHEN, *Anal. Biochem.*, 5 (1963) 99.
4 A. A. PATTI, P. BONANNO, T. F. FRAWLEY AND A. A. STEIN, *Acta Endocrinol.*, Suppl. 77 (1963).

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J. Chromatog., 12 (1963) 242–245

Notes

Devices for continuous development and sample application in preparative thin-layer chromatography

The resolution of closely related substances by thin-layer chromatography may often be improved by decreasing the polarity of the solvent system to a point where the R_F values are less than 0.1. Either repeated or continuous development is then required to obtain appreciable mobilities and complete separation. Repeated development has the advantage of being technically simple and, because the lower parts of the zones are reached first by the new solvent front, tailing effects are reduced. However, a disadvantage is the long drying time required between developments when relatively thick layers are used for preparative work.

In paper chromatography, continuous development is usually carried out by the descending overflow technique. Methods for descending development of thin-layer chromatograms have been described[1,2], but the zones are broader than those obtained by ascending development[2] and special apparatus is required. Two methods of continuous development based on evaporation of the solvent from the terminal edge of the plate have been reported[2,3]. BRENNER AND NIEDERWIESER[3] used a hori-

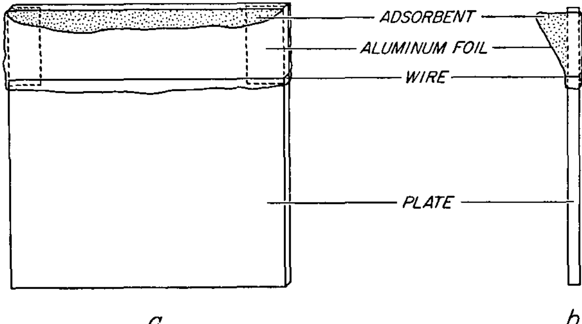

Fig. 1. Device for continuous development of thin-layer chromatograms, (a) front view; (b) side view.

zontal development technique in which solvent is fed to the plate by a paper wick, evaporation from the surface of the plate being prevented by an overlaid glass plate. This method does not require a developing chamber and it has been used successfully with preparative plates, but the development time is about twice that required for ascending development[4]. ZÖLLNER AND WOLFRAM[2] used ascending development in a chamber kept partly open to allow evaporation from the top edge, while preventing evaporation in the lower part of the plate with solvent-soaked paper. This arrangement leads to changes in the composition of solvent mixtures due to differential evaporation and is therefore best suited for use with a single solvent.

We have been using a method of continuous development which requires no

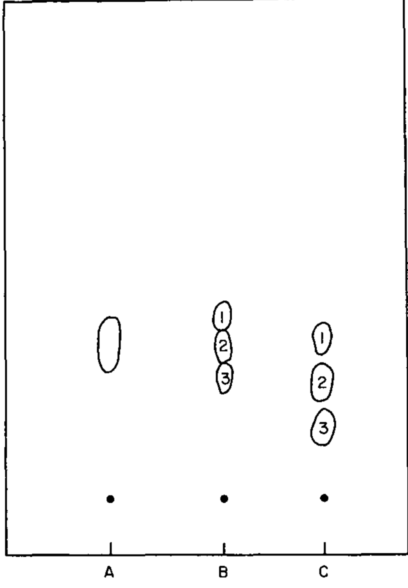

Fig. 2. Separation of 1 μg each of β-sitosterol acetate (1), cholesterol acetate (2), and stigmasterol acetate (3), by (A) single 10 cm development with hexane–ether (80:20) for 9 min; (B) repeated development (five times) with hexane–ether (94:6) for 60 min; (C) continuous development with hexane–ether (97:3) for 120 min; on Anasil B plates, 275 μ thick.

special apparatus, can be used with plates of different sizes, and is adaptable to either analytical or preparative thin-layer chromatography. It consists essentially of a trough of aluminum foil, filled with loose adsorbent, which is attached to the top of the plate. The trough is tied to the plate with a thin wire and the sides of the aluminum foil are folded around the edges of the plate (Fig. 1). Enough adsorbent can be placed in the trough to allow Silica Gel G* plates to be developed overnight, although 6–8 h is usually sufficient. The adsorbent can, of course, be reused after drying.

Fig. 2 shows the differences in separation of three sterol acetates by the usual ascending technique, by repeated development, and by continuous development It will be observed that, although continuous development causes the spots to become more diffuse, the separation is better than by the other two methods. It is interesting that cholesterol acetate and β-sitosterol acetate, differing only by an ethyl group in a saturated side chain, can be separated by adsorption chromatography on Anasil**, but not on Silica Gel G[5].

We have found this method of continuous development especially useful in preparative thin-layer chromatography, based on the procedures of HONEGGER[4] with the following modifications. Rhodamine 6 G*** (0.1 mg per plate) is dissolved in the water used to prepare the slurry of Silica Gel G. The plates, usually 1 mm thick, are first developed with acetone to move extractable material to the top edges. The solvent systems (100–250 ml) are allowed to equilibrate for 30–60 min in the developing chambers* (30.5 × 9.9 × 27.6 cm), which are lined completely with Whatman No. 3 MM paper. After development and drying, the bands are located and marked under short-wave ultraviolet light. Less than 1 mg of compound spread. across a 200 × 200 mm Silica Gel G plate, 1 mm thick, can be detected by this method. The bands are scraped off the plate, placed in a chromatographic column, and eluted with acetone. Enough of the dye is eluted to produce a light yellow color, but this is easily removed in the further purification steps.

In many cases a micro pipet is satisfactory for the application of the sample solution along the starting line. However, if the mobilities of the compounds are markedly dependent on their concentrations, the zones will be irregular, as it is very difficult to achieve even distribution across the plate by this method. We have found an easily constructed applicator to be useful in such instances. This device, shown in Fig. 3, is assembled as follows. One side of a 75 × 50 × 1 mm microscope slide is covered lengthwise with pressure-sensitive tape§ to a thickness of about 0.5 mm, except for an area of about 18 × 75 mm. Another slide is placed over it and secured by tape, making certain that the lower edges of both slides are exactly even and free of imperfections. Two such applicators are mounted in a holder, prepared as follows. Tape is wound around the ends of two glass rods, 200 mm long, to a thickness of 2 mm. The rods are then bound together at the ends with tape. The applicators are supported in this holder by metal clips in such a way that they can move freely up and down but cannot fall through; a narrow piece of tape wound around the middle of the holder keeps them separated.

* Brinkmann Instruments Inc., Great Neck, New York.
** Analytical Engineering Laboratories, Inc., Hamden, Conn.
*** Fisher Scientific Co.
§ Time Tape, Professional Tape Co. Inc., Riverside, Ill.

The lower edges of the applicators are now dipped into the solution of sample to be applied. The solution is taken up by capillary action, which may be regulated by varying the distance between the slides with a suitable number of layers of tape. The applicator is then positioned just above the starting line on the plate and the

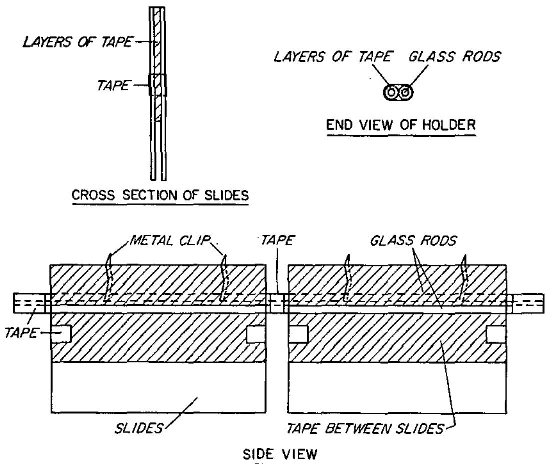

Fig. 3. Sample applicator for preparative thin-layer chromatography.

glass rods, held by the ends, are *rapidly* dropped so that the lower edges of the slides rest flat on the plate. If this operation is carried out rapidly enough, the edges will rest evenly on the plate and the solution will flow out in two even bands. There is little or no disturbance of the adsorbent surface, even with the more fragile Anasil layers. This operation can be repeated as many times as necessary to transfer all of the sample to the plate.

To test the efficiency of the application and elution steps, 50 mg of cholesterol acetate was applied to a 200 × 200 mm Silica Gel G plate, 1 mm thick, using the apparatus described above. After development with dichloromethane and drying, the zone was located, scraped off the plate, and eluted with acetone, 99 mole % pure* (about 50 ml). Evaporation of the acetone left 48 mg of cholesterol acetate, homogeneous by analytical thin-layer chromatography.

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1 L. BIRKOFER, C. KAISER, H.-A. MEYER-STOLL AND F. SUPPAN, *Z. Naturforsch.*, 17b (1962) 352.
2 N. ZÖLLNER AND G. WOLFRAM, *Klin. Wochschr.*, 40 (1962) 1098.
3 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 15 (1961) 237.
4 C. G. HONEGGER, *Helv. Chim. Acta*, 45 (1962) 1409.
5 R. D. BENNETT AND E. HEFTMANN, *J. Chromatog.*, 9 (1962) 359.

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* Fisher Scientific Co.

Nachweis von Acetessigsäure und Aceton im Harn mit Hilfe der Dünnschichtchromatographie

Prinzip

Aceton und Acetessigsäure werden in ihre 2,4-Dinitrophenylhydrazone übergeführt, die sich auf Cellulosepulver trennen lassen.

Verfahren

10.0 g Cellulosepulver (MN 300 Ac, Macherey und Nagel, Düren) werden mit 50.0 ml Methanol und 5.0 ml dest. Wasser ca. 1 Minute lang im Starmix homogenisiert. (Ansatz für 5 Platten 200 × 200 mm*). Die behandelten Platten werden nach dem Vortrocknen an der Luft 5–10 Minuten lang bei 60° getrocknet.

Als Elutionsgemisch wurden verwandt:

I. Methanol–Wasser–Ammoniakflüssigkeit (25 %ig), (90:10:3 v/v).

II. *n*-Propanol–Ammoncarbonatlösung (2 Vol. Teile 10 %ige Ammoncarbonatlösung und 1 Vol. Teil 5*N* Ammoniak) (2.5 : 1 v/v).

Arbeitsvorschrift

5.0 ml klarer, bzw. klar filtrierter Harn werden tropfenweise solange mit 2,4-Dinitrophenylhydrazinlösung (0.4 %ig in 2 *N* HCl) versetzt, bis sich die Trübung nicht mehr verstärkt. Nach 1/2-stündigem Stehen in der Kälte schüttelt man mit 1–2 ml Aethylacetat aus, trägt die organische Phase auf die Platten auf und chromatographiert bei Zimmertemperatur unter Normalsättigung der Kammer. Die Vergleichslösungen von Aceton und Acetessigsäure werden auf die gleiche Weise bereitet. Laufzeit 30–45 Minuten, Trennstrecke 100 mm.

Nach dem Trocknen der entwickelten Platten an der Luft erscheinen die Dinitrophenylhydrazone im Tageslicht als gelbe, im U.V. Licht als dunkle Flecken.

Die aufgetragenen Substanzmengen lagen zwischen 1–5 γ. Die R_F-Werte sind in Tabelle I angeführt.

TABELLE I

R_F-WERTE DER 2,4-DINITROPHENYLHYDRAZONE IN 2 SYSTEMEN

2,4-Dinitrophenylhydrazon	*R_F-Werte*	
	Elutionsmittel I	*Elutionsmittel II*
Aceton	0.48	0.52
Acetessigsäure	0.82	0.91

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MELANIE RINK
SIGRID HERRMANN

Eingegangen den 11. Februar 1963

* Desaga Grundausrüstung.

A new color reaction with potassium permanganate and bromophenol blue on thin-layer chromatograms

A new color reaction was devised in the course of studies on thin-layer chromatography of antibiotics.[1] When organic compounds are detected by means of potassium permanganate alone, the sensitivity is not very high and the color becomes vague with passage of time. The authors found that, if 0.2 % aqueous bromophenol blue solution is sprayed about 10 or 15 min after treatment with 0.5 % aqueous potassium permanganate solution at room temperature, a blue or greenish blue color appears depending on the degree of acidity of the reaction products resulting from the oxidation, and that the coloration lasts more than a month with enough contrast to be directly photographed. Furthermore the sensitivity is enhanced more than ten fold.

For example, for the detection of amaromycin, a macrolide antibiotic, by potassium permanganate alone, more than 1 μg was necessary. When the chromatogram was sprayed with bromophenol blue solution after the treatment with permanganate, less than 0.1 μg of the material was sufficient for detection. Phenolphthalein, litmus, or crystal violet were tried but without success. Thymol blue could be used instead of bromophenol blue when the reaction products had a higher acidity.

All antibiotics studied by thin-layer chromatography by the authors[1] were detected by means of this color reaction, except antimycin A, griseofulvin, amidinomycin, and fradiomycin (neomycin); these were detected with a mixture of 1 % methyl red and bromothymol blue solution instead of bromophenol blue solution.

The color reaction described in this paper can be generally applied to the detection of other organic substances, for instance, some amino acids, peptides, monosaccharides and unsaturated organic compounds. Results of the color reaction with sugars, organic acids, amino acids and others are shown in Table I. Since most of the known antibiotics are polyfunctional, we can expect that at least one of the functional

TABLE I

COLOR REACTIONS OF VARIOUS ORGANIC COMPOUNDS WITH POTASSIUM PERMANGANATE AND BROMOPHENOL BLUE

Tests were run with two kinds of plates: Silicagel (Wölm) and Alumina (pH 3.6) (Wölm)

	Potassium permanganate reaction	*Bromophenol blue coloration*
Octanol	—	no spot
Glycerol	+	pH 4.0–4.2
Glucose	+*	pH 4.4–4.8
Xylose	+*	pH 4.4–4.8
Galactose	+*	pH 4.4–4.8
Fructose	+*	pH 4.4–4.8
Sucrose	—	no spot
Lactose	—	no spot
Maltose	—	no spot
Stearic acid	—	pH 2–3**
Succinic acid	+	pH 2–3**
Tartaric acid	+	pH 2–3**
Citric acid	+*	pH 2–3**
Glycine	±	practically no spot
Alanine	±	practically no spot

(continued on p. 251)

TABLE I (*continued*)

	Potassium permanganate reaction	Bromophenol blue coloration
Phenylalanine	±	practically no spot
Leucine	±	practically no spot
Valine	—	no spot
L-Cysteine	—	no spot
Aspartic acid	±	practically no spot
Glutamic acid	±	practically no spot
Lysine	±	practically no spot
Threonine	+*	pH 4.2–4.4
Serine	+*	pH 4.4
Proline	+*	pH 2–3**
Hydroxyproline	+*	pH 4.8
Methionine	+	pH 4.2–4.4
Tyrosine	+*	pH 4.0
Tryptophan	+	pH 4.4
Arginine·HCl	+*	pH 4.4
Histidine·HCl	+*	pH 4.0
Cholesterol	+*	pH 4.4
Acetylacetone	+	pH 4.4–4.8
Crotonic acid	+	pH 4.6
Xylol	—	no spot
Phenol	—	no spot
β-Naphthol	+*	pH 4.4–4.8
Anthraquinone	—	no spot
Anthrone	+	pH 3.6–4.0
Benzoic acid	±	pH 2–2.4**
p-Dimethylamino-benzaldehyde	+	pH 4.4–4.8
Benzidine	green	——
o-Dianisidine	green	——

* Spot appeared slowly.
** Detected by both bromophenol blue and thymol blue.

groups will react with potassium permanganate to give a detectable spot on the plate. Substances indifferent to potassium permanganate at room temperature, such as simple aromatic substances, disaccharides or straight chain saturated aliphatic acids cannot be detected. Depending on their acidity the acids can be detected either by thymol blue or by bromophenol blue.

The authors express their deep gratitude to Dr. H. UMEZAWA, Director of this institute, for his kind and helpful guidance and encouragement.

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[1] T. IKEKAWA, F. IWAMI, E. AKITA AND H. UMEZAWA, *J. Antibiotics (Tokyo)*, A 16 (1963) 56.

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A simple apparatus for the application of material to paper strips for chromatography

In the course of investigations requiring the application of large numbers of samples to paper strips for chromatography, no previously described method has been found satisfactory for holding and drying the strips during sample application. The following apparatus has been extremely useful over a period of several years' trial and is described herein with the hope that it may be of some value to other investigators faced with a similar problem.

The basic requirements are a block of wood with two holes drilled in it at right angles to each other, a few pieces of thin flat aluminum, and an aluminum tube. The construction of the apparatus is apparent from Fig. 1. A common laboratory heat

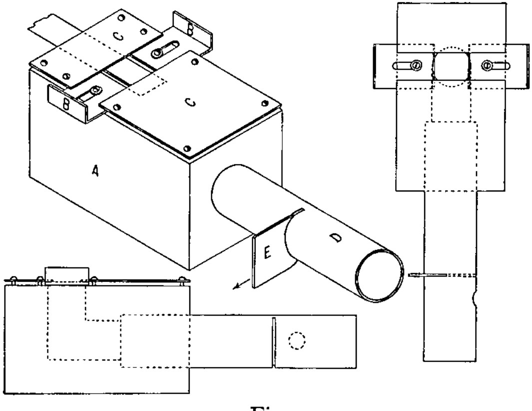

Fig. 1.

gun fits into the open end of the aluminum tube (D) to provide either warm or cold forced air which goes through the wooden block (A) directly to the underside of the paper strip (shown in place in the upper left hand figure) and out through the open sides between the aluminum plate (C) and the block. The paper strip is held in place underneath the aluminum plate by adjustable side pieces of aluminum (B) to allow the use of differing widths of paper. A nonconducting hand rest of wood or paper can be placed over the top of the aluminum plate to facilitate application of the material if necessary. A damper of thin aluminum (E) is also used to control the flow of air to the paper.

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Dispositif d'extraction continue des papiers pour chromatographie

Il arrive fréquemment lorsque l'on désire effectuer des chromatographies quantitatives sur papier que l'on soit obligé de purifier celui-ci, soit pour enlever les traces d'ions minéraux qui ont pu rester au sein de la pate de cellulose, soit pour enlever d'autres corps réducteurs ou impuretés de nature organique qui peuvent s'être déposés sur le papier.

C'est ainsi que nous avons été amenés à devoir procéder à la purification des papiers pour chromatographie bidimensionnelle des tocophérols selon la technique de GREEN, MARCINKIEWICZ ET WATT[1,2]. Il est notamment recommandé pour avoir un "blanc" très faible de procéder à un lavage du papier par l'alcool méthylique dans un soxhlet ou à défaut à l'aide d'éprouvettes.

Il est extrêmement difficile de se procurer des extracteurs continus type soxhlet ou Kumagawa ayant un panier d'extraction de 30 cm de hauteur pour y loger les feuilles de papier. L'emploi d'éprouvettes ne réalise qu'un lavage partiel et nécessite une grande quantité de solvant. Nous avons utilisé un montage simple, fonctionnant en continu, et qui permet de purifier le papier en 4 ou 5 heures.

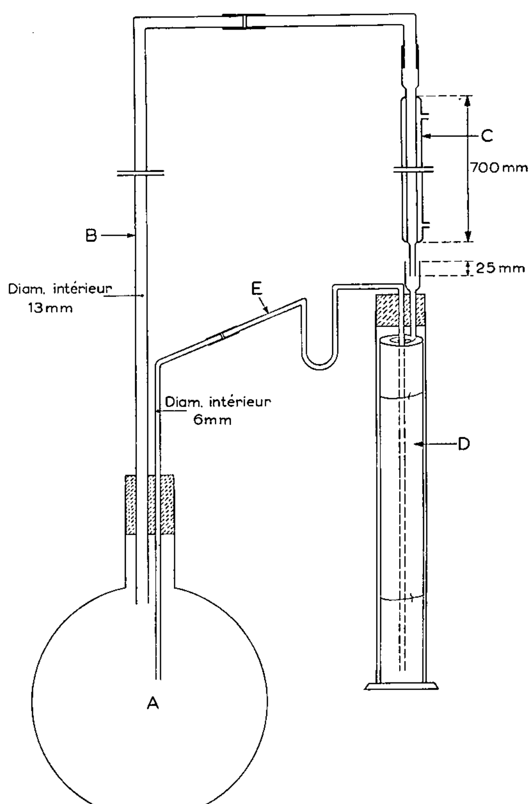

Fig. 1. Schéma du montage réalisé comprenant: A = Ballon en pyrex de 2 litres; B = Conduit de vapeur; C = Réfrigérant; D = Extracteur; E = Siphon.

Les jonctions ont été effectuées à l'aide de tuyau en matière plastique, mais il est bien évident q'nue installation à l'aide de rodages sphériques permettrait de donner une certaine maniabilité à l'ensemble, tout en éliminant l'emploi des tuyaux en matière plastique.

Bien que le fonctionnement de ce dispositif soit très simple, il nous semble utile de préciser quelques détails:

1. Le chauffage doit être effectué avec un chauffe-ballon électrique permettant une ébullition très rapide. La régulation de cette ébullition à l'aide de pierre ponce donne entière satisfaction.

2. L'extracteur peut être obturé par un bouchon de caoutchouc sans aucun inconvénient, du fait de la présence d'une couche d'air qui s'interpose entre le liquide et le bouchon au cours du remplissage.

3. L'extrémité libre du réfrigérant plonge dans l'entonnoir de l'extracteur, ce qui permet au cours du remplissage l'établissement d'une colonne de liquide dans le tube du réfrigérant et l'amorçage du siphon.

4. Le tube en U disposé sur le siphon évite que la vapeur du solvant ne reflue dans l'extracteur par cette voie, la condensation des premières gouttes laisse dans le tube une quantité suffisante de liquide pour s'opposer au passage de la vapeur.

5. Les feuilles de papier pour chromatographie sont enroulées en cylindre et maintenues à l'aide de deux ligatures en nylon. Une douzaine de feuilles 30 cm × 30 cm peuvent être extraites en même temps.

6. Dans le cas où l'on désire utiliser comme solvant un liquide à point d'ébullition élevé, il sera nécessaire de disposer autour du conduit de vapeur un cordon électrique chauffant qui permettra un fonctionnement plus rapide.

En dehors de sa facilité de montage à l'aide d'un matériel que l'on rencontre dans tous les laboratoires, il est intéressant de signaler que l'appareil peut être adapté à la largeur des feuilles à extraire par l'emploi d'un extracteur (éprouvette) de hauteur appropriée.

1 J. Green, S. Marcinkiewicz et P. R. Watt, *J. Sci. Food Agr.*, 6 (1955) 274.
2 Analytical Methods Committee, Report prepared by the Vitamine E Panel, *Analyst*, 84 (1956) 356.

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Surface active agents in paper chromatography

A recent note by ORME-JOHNSON AND SKINNER[1] on the separation of amino acids with butanol containing the liquid ion exchanger Amberlite LA-2 prompts us to describe work carried out along similar lines[2]. It was thought interesting to study the possible effects that surface active agents can have in various paper chromatographic problems, with a view to utilising paper chromatography as a simple orientating method in the study of foam adsorption and similar problems.

(1) The effect of additions of surface active agents to the solvent in paper chromatography

Paper chromatography was carried out using the solvent butanol–water–acetic acid (10:8:2) to which various amounts of cetyltrimethylammonium bromide, sodium lauryl sulphonate or "Neutronix 675" a neutral polyethyleneglycol surface active agent were added. The substances chromatographed were some inorganic anions and cations and a series of dyestuffs (those permitted in Italy as food colours).

The results are shown in Tables I, II and III. An increase in the R_F values is obtained in those cases where the "exchanger" (or surface active agent) has the charge opposite to that of the ion chromatographed and the effect is proportional to the amount of surface active agent added.

It is thus possible to remove an anion from a mixture of cations and anions or vice-versa by suitable additions of charged surface active agents to partition solvents.

TABLE I

R_F VALUES OF SOME DYESTUFFS AND INORGANIC IONS IN BUTANOL–ACETIC ACID–WATER WITH ADDITIONS OF CETYLTRIMETHYLAMMONIUM BROMIDE

Paper: Whatman No. 1. Method: ascending development. Solvent: butanol–acetic acid–water (100:20:80), equilibrated, and the amounts of cetyltrimethylammonium bromide indicated in the table added to the upper layer

Substance	*Grams of cetyltrimethylammonium bromide added to the solvent*				
	1.0	*0.5*	*0.1*	*0.05*	*0*
Iodide	0.75	0.59	0.34	0.31	0.31
Thiocyanate	0.77	0.53	0.47	0.46	0.41
Azorubin	0.92	0.82	0.52	0.37	0.31
Echtrot E	0.86	0.72	0.40	0.32	0.32
Amaranth	0.35	0.13	0.08	0.04	0.03
Ponceau 4R	0.79	0.53	0.13	0.10	0.08
Ponceau 6R	0.52	0.06	0	0	0
Scharlach GN	0.70	0.59	0.29	0.26	0.26
Erythrosine	0.90	0.90	0.90	0.89	0.86
Sunset Yellow	0.51	0.17	0.14	0.12	0.14
Tartrazine	0.09	0.02	0.04	0.06	0.05
C.I. Acid Yellow	0.06	0.02	0.02	0.02	0.02
	0.20	0.16	0.16	0.08	0.09
Indigo Carmine	0.08	0.06	0.06	0.04	0.05
C.I. Acid Blue 3	0.70	0.62	0.52	0.42	0.45
C.I. Food Black 1	0.03	0	0	0	0

TABLE II

R_F VALUES OF SOME INORGANIC IONS AND DYESTUFFS IN BUTANOL–ACETIC ACID–WATER WITH ADDITIONS OF SODIUM LAURYLSULPHONATE

Paper: Whatman No. 1. Method: ascending development. Solvent: butanol–acetic acid–water (100:20:80), equilibrated, and the amounts of sodium laurylsulphonate indicated in the table added to the upper layer

Substance	*Grams of sodium laurylsulphonate added to the solvent*				
	1.0	*0.5*	*0.1*	*0.05*	*0*
Cu (II)	0.58	0.44	0.28	0.30	0.23
Ni (II)	0.65	0.45	0.19	0.17	0.14
Co (II)	0.65	0.44	0.21	0.17	0.14
Scharlach GN	0.15	0.16	0.20	0.24	0.26
Erythrosine	0.89	0.89	0.92	0.91	0.86
Sunset Yellow	0.09	0.12	0.16	0.16	0.14
Tartrazine	0	0.01	0.09	0.06	0.05
C.I. Acid Yellow 3	0.15	0.12	0.11	0.16	0.09
	0.03	0.01	0.02	0.02	0.02
Indigo Carmine	0.01	0.02	0.02	0.08	0.05
C.I. Acid Blue 3	0.42	0.40	0.43	0.39	0.45
C.I. Food Black 1	0	0	0	0	0

TABLE III

R_F VALUES OF SOME INORGANIC IONS AND DYESTUFFS IN BUTANOL–ACETIC ACID–WATER WITH ADDITIONS OF NEUTRONIX 675 (A NEUTRAL SURFACE ACTIVE AGENT)

Paper: Whatman No. 1. Method: ascending development. Solvent: butanol–acetic acid–water (100:20:80), equilibrated, and the amounts of Neutronix 675 indicated in the table added to the upper layer

Substance	*Grams of Neutronix 675 added to the solvent*				
	1.0	*0.5*	*0.1*	*0.05*	*0*
Iodide	0.25	0.31	0.32	0.27	0.31
Thiocyanate	0.45	0.46	0.43	0.43	0.42
Cu (II)	0.25	0.21	0.25	0.23	0.23
Ni (II)	0.13	0.17	0.17	0.15	0.14
Co (II)	0.13	0.17	0.20	0.13	0.14
Azorubin	0.27	0.34	0.26	0.27	0.30
Echtrot E	0.25	0.28	0.26	0.23	0.31
Amaranth	0.02	0.02	0.01	0	0.04
Ponceau 4R	0.08	0.06	0.04	0.04	0.07
Ponceau 6R	0	0	0	0	0
Scharlach GN	0.24	0.25	0.25	0.20	0.27
Erythrosine	0.90	0.90	0.89	0.90	0.88
Sunset Yellow	0.11	0.08	0.09	0.08	0.17
Tartrazine	0.02	0	0	0.02	0.02
C.I. Acid Yellow 3	0.12	0.10	0.04	0.04	0.08
	0.02	0.02	0	0.02	0.02
Indigo Carmine	0.04	0.03	0.04	0.05	0.05
C.I. Acid Blue 3	0.46	0.39	0.35	0.36	0.44
C.I. Food Black 1	0	0	0	0	0

It should also be possible to gain information about the charge of an unknown substance by this method.

(2) The effect of surface active agents on the R_F value of substances when the surface active agent is present in the sample to be analysed

Artificial mixtures of solutions of surface active agents and dyestuffs or inorganic ions were prepared, placed on paper and developed with butanol–water–acetic acid (10:8:2).

As shown in Tables IV, V and VI as well as Fig. 1, two kinds of results may be obtained. The presence of the surface active agent of the opposite charge to that of the substance may produce either two distinct spots with the same substance or an elongated trail. Neutral or equally charged surface active agents have no effect.

The double spot phenomenon may be explained if we consider that the bulk of the surface active agent moves probably in a micellar form and that these micelles are capable of adsorbing some of the oppositely charged substance or alternatively that very strong ion association complexes are formed between the surface active agent and the ion.

The effect is of importance when substances *e.g.* dyestuffs are to be identified by their R_F values. This is clearly impossible where surface active agents are present in a sample.

(3) The effect of surface active agents in adsorption chromatograms on cellulose paper

Preliminary work with solvents containing surface active agentsindicated that these were adsorbed on the cellulose surface as are also the more insoluble "liquid ion exchangers" (see CERRAI AND TESTA[3]). In order to avoid a gradient effect due to a

(text continued p. 261)

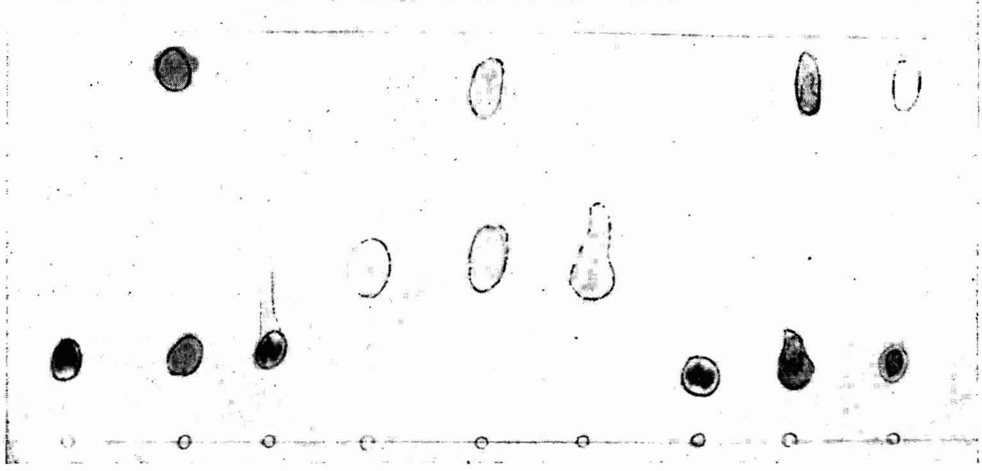

Fig. 1. Chromatogram of some dyestuffs mixed with cetyltrimethylammonium bromide. Solvent: butanol–acetic acid–water (100:20:80). From left to right: Scharlach GN; Scharlach GN mixed with a 10% solution of cetyltrimethylammonium bromide; Scharlach GN mixed with a 1% solution of cetyltrimethylammonium bromide; C.I. Acid Blue 3 alone and mixed with 10% and 1% cetyltrimethylammonium bromide solutions; Echtrot E alone and mixed with 10% and 1% cetyltrimethylammonium bromide. Bottom line: line of application of spots. Top line: liquid front.

TABLE IV

R_F VALUES OF SOME INORGANIC IONS AND DYESTUFFS WHEN A MIXTURE OF THE SUBSTANCE WITH 10% OR 1% SOLUTIONS OF CETYLTRIMETHYLAMMONIUM BROMIDE ARE CHROMATOGRAPHED IN BUTANOL–ACETIC ACID–WATER

Paper: Whatman No. 1. Method: ascending development. Solvent: butanol–acetic acid–water (100:20:80) (upper phase).

Substance	*Concentration of cetyltrimethylammonium bromide mixed with the substance to be chromatographed*		
	10%	*1%*	*0*
Iodide	0.92, 0.33	0.46	0.30
Thiocyanate	0.90, 0.44	0.44	0.40
Azorubin	0.88, 0.30	0.88, 0.38	0.30
Echtrot E	0.85, 0.19	0.89, 0.29	0.31
Amaranth	0.85, 0.15	0.04, Tail	0.04
Ponceau 4R	0.80, 0.10	0.06, Tail	0.07
Ponceau 6R	0.86	0	0
Scharlach GN	0.86, 0.30	0.90, 0.39	0.27
Erythrosine	0.88	0.88	0.88
Sunset Yellow	0.83, 0.17	0.17	0.17
Tartrazine	0.24	0.07	0.02
C.I. Acid Yellow 3	0.79, 0.20	0.19	0.08, 0.02
Indigo Carmine	0.11	0.02	0.02
C.I. Acid Blue 3	0.83, 0.46	0.46	0.44
C.I. Food Black 1	0 Tail	0	0

TABLE V

R_F VALUES OF SOME INORGANIC IONS AND DYESTUFFS WHEN A MIXTURE OF THE SUBSTANCE WITH 10% OR 1% SOLUTIONS OF SODIUM LAURYLSULPHONATE ARE CHROMATOGRAPHED IN BUTANOL–ACETIC ACID–WATER

Paper: Whatman No. 1. Method: ascending development. Solvent: butanol acetic–acid–water (100:20:80) (upper phase).

Substance	*Concentration of sodium laurylsulphonate mixed with the substance to be chromatographed*		
	10%	*1%*	*0*
Cu (II)	0.22 (with trail)	0.16	0.20
Ni (II)	0.26 (with trail)	0.07	0.13
Co (II)	0.22 (with trail)	0.08	0.14
Scharlach GN	0.25	0.30	0.25
Erythrosine	0.89	0.90	0.89
Sunset Yellow	0.15	0.15	0.15
Tartrazine	0.06	0.04	0.03
C.I. Acid Yellow 3	0.08	0.09	0.10
	0.05	0.06	0.02
Indigo Carmine	0.06	0.07	0.05
C.I. Acid Blue 3	0.43	0.44	0.45
C.I. Food Black 1	0.09	0.09	0

TABLE VI

R_F VALUES OF SOME INORGANIC IONS AND DYESTUFFS WHEN A MIXTURE OF THE SUBSTANCE WITH 10% SOLUTIONS OF NEUTRONIX 675 (A NEUTRAL SURFACE ACTIVE AGENT) IS CHROMATOGRAPHED IN BUTANOL–ACETIC ACID–WATER

Paper: Whatman No. 1. Method: ascending development. Solvent: butanol–acetic acid–water (100:20:80).

Substance	*Concentration of Neutronix 675 mixed with the substance to be chromatographed*		
	10%	*1%*	*0*
Iodide	0.27	0.27	0.31
Thiocyanate	0.36	0.37	0.40
Cu (II)	0.25	0.24	0.23
Ni (II)	0.16	0.15	0.14
Co (II)	0.15	0.15	0.15
Azorubin	0.21	0.22	0.30
Echtrot E	0.22	0.23	0.31
Amaranth	0.01	0	0.03
Ponceau 4R	0.03	0.05	0.06
Ponceau 6R	0	0	0
Scharlach GN	0.20	0.21	0.23
Erythrosine	0.87	0.90	0.90
Sunset Yellow	0.14	0.14	0.15
Tartrazine	0.02	0.03	0.02
C.I. Acid Yellow 3	0.05	0.03	0.09
	0.02	0.03	0.02
Indigo Carmine	0.02	0.04	0.05
C.I. Acid Blue 3	0.44	0.44	0.44
C.I. Food Black 1	0	0	0

TABLE VII

R_F VALUES OF SOME INORGANIC IONS AND DYESTUFFS ON PAPER IMPREGNATED WITH VARIOUS AMOUNTS OF CETYLTRIMETHYLAMMONIUM BROMIDE

Paper: Whatman No. 1 dipped in aqueous solutions of cetyltrimethylammonium bromide (concentrations indicated below) and air dried. Method: ascending development. Solvent: water–acetic acid (90:10).

Substance	*Concentration of cetyltrimethylammonium bromide used for impregnating the paper*					
	1%	*0.5%*	*0.1%*	*0.05%*	*0.01%*	*0*
Iodide	0.54	0.62	0.71	0.70	0.76	0.81
Thiocyanate	0.49	0.62	0.67	0.68	0.76	0.80
Azorubin	1.0,0	1.0,0	1.0,0	1.0,0	0.16	0.20
Echtrot E	1.0,0	1.0,0	1.0,0	1.0,0	0.25	0.29
Amaranth	1.0,0	1.0,0	1.0,0	0.34	0.27	0.36
Ponceau 4R	1.0,0	1.0,0	1.0,0	1.0,0	0.36	0.74
Ponceau 6R	1.0,0	1.0,0	1.0,0	1.0,0	0.48	0.90
Scharlach GN	1.0,0	1.0,0	1.0,0	0.18	0.39	0.60
Erythrosine	1.0,0	1.0,0	1.0,0	0	0	0
Sunset Yellow	1.0,0	1.0,0	1.0,0.17	0.25	0.25	0.62
Tartrazine	1:0,0	1.0,0	1.0,0.17	0.15	0.25	0.63
C.I. Acid Yellow 3	1.0,0	1.0,0	1.0,0	0.18	0.21	0.38
Indigo Carmine	1.0,0	1.0,0	1.0,0	0.15	0.25	0.38
C.I. Acid Blue 3	1.0	1.0	1.0,0.53	0.64	0.65	0.85
C.I. Food Black 1	1.0,0	1.0,0	1.0,0.04	0.03	0.04	0.12

TABLE VIII

R_F VALUES OF SOME INORGANIC IONS AND DYESTUFFS ON PAPER IMPREGNATED WITH VARIOUS AMOUNTS OF SODIUM LAURYLSULPHONATE

Paper: Whatman No. 1 dipped in aqueous solutions of sodium laurylsulphonate (concentrations indicated below) and air dried. Method: ascending development. Solvent: water–acetic acid (90:10)

Substance	*Concentration of sodium laurylsulphonate used for impregnating the paper*				
	1%	*0.5%*	*0.1%*	*0.05%*	*0*
Cu (II)	1.0	1.0	1.0	1.0	1.0
Ni (II)	1.0	1.0	1.0	1.0	0.86
Co(II)	1.0	1.0	1.0	1.0	0.91
Scharlach GN	0.62	0.58	0.60	0.60	0.61
Erythrosine	0.90	0.90	0	0	0.90
	0	0			
Sunset Yellow	0.54	0.50	0.52	0.62	0.59
Tartrazine	0.61	0.59	0.60	0.63	0.63
C.I. Acid Yellow 3	0.55	0.55	0.54	0.38	0.38
	0.35	0.35	0.33		
Indigo Carmine	0.33	0.28	0.30	0.36	0.38
C.I. Acid Blue 3	1.0	1.0	0.89	0.85	0.85
C.I. Food Black 1	0.12	0.13	0.12	0.12	0.12

TABLE IX

R_F VALUES OF SOME INORGANIC IONS AND DYESTUFFS ON PAPER IMPREGNATED WITH VARIOUS AMOUNTS OF NEUTRONIX 675 (A NEUTRAL SURFACE ACTIVE AGENT)

Paper: Whatman No. 1 dipped into an aqueous solution of Neutronix 675 (concentrations indicated below) and air dried. Method: ascending development. Solvent: water–acetic acid (90:10).

Substance	*Concentration of Neutronix 675 used for impregnating the paper*			
	1%	*0.5%*	*0.1%*	*0*
Iodide	1.0	0.86	0.89	0.81
Thiocyanate	1.0	0.85	0.86	0.82
Cu (II)	1.0	1.0	1.0	1.0
Ni (II)	1.0	1.0	1.0	0.88
Co (II)	1.0	1.0	1.0	0.91
Azorubin	0.19	0.20	0.24	0.18
Echtrot E	0.26	0.25	0.27	0.29
Amaranth	0.33	0.32	0.34	0.36
Ponceau 4R	0.96	0.95	0.80	0.75
Ponceau 6R	0.99	0.96	0.90	0.90
Scharlach GN	0.59	0.59	0.56	0.60
Erythrosine	0.91	0.90	0.88	0.86
Sunset Yellow	0.37	0.40	0.49	0.62
Tartrazine	0.70	0.62	0.65	0.63
C.I. Acid Yellow 3	0.39	0.39	0.38	0.39
Indigo Carmine	0.40	0.38	0.36	0.38
C.I. Acid Blue 3	0.87	0.85	0.90	0.85
C.I. Food Black 1	0.13	0.10	0.11	0.12

liquid zone moving over paper poor in surface active agent (which could occur if the surface active agent were present in the solvent), we impregnated the papers by dipping them into aqueous solutions of the surface active agent and drying in air. The papers were then developed with aqueous solvents *e.g.* water–acetic acid (9:1).

Results with inorganic ions and dyestuffs are shown in Tables VII, VIII and IX. In general, above a certain concentration of the surface active agent, the dyestuff separates into two spots, one strongly adsorbed on the point of origin and another on the liquid front. Below that concentration the R_F value of the dyestuff is lower than the value obtained when no surface active agent is present. This effect is only observed when the surface active agent has a charge opposite to that of the substance chromatographed.

The phenomenon may be explained by considering that a certain amount of surface active agent can adsorb on the paper thus forming a solid ion exchanger with strong adsorptive power. The excess of surface active agent moves with the liquid front, probably in a micellar state and adsorbs some of the dyestuff into the micelles. There is a fair correlation between the concentrations at which the two spots form and the maxima of the surface tension–concentration curves for the surface active agent. Below the maximum surface tension in water, two spots do not form on the chromatogram.

Inorganic ions *e.g.* iodide and thiocyanate do not form double spots, the adsorption on the micelles perhaps not being strong enough. Their R_F values change, however, with the concentration of the (cationic) surface active agent, since adsorption is greater with increased concentration.

In such adsorption chromatograms, the charge on the surface active agent seems of great importance although one would expect the surface of the paper to be altered considerably even when covered by a neutral surface active agent.

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[1] W. H. ORME-JOHNSON AND C. G. SKINNER, *J. Chromatog.*, 11 (1963) 549.
[2] E. FARULLA, *Doctorate Thesis*, University of Rome, completed summer 1962.
[3] E. CERRAI AND C. TESTA, *J. Chromatog.*, 5 (1961) 442.

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Estimation of phosphorus on paper chromatograms

The sensitive method of phosphorus analysis described by BARTLETT[1] is particularly suitable for the estimation of organophosphorus compounds on paper chromatograms and electrophoretograms. Reasonable accuracy may be achieved without using special techniques or high individual spot concentrations. The method described here has been found to yield results accurate to better than $\pm$ 3 % with spots which contain from 0.5 to 15 μg of phosphorus.

The chromatogram, developed in purified solvents, is dried in the usual way and sprayed lightly with HANES–ISHERWOOD reagent[2]. After about 30 min the phosphorus-containing areas are detected by irradiating the paper with a high intensity mercury vapour lamp[3] (Hanovia 501/1, and the spots are then excised, allowing a small border round each to ensure full recovery of the phosphorus. A control "blank" of equal area, is cut from the same chromatogram, preferably from about the same R_F position. The pieces are placed in separate Pyrex tubes (120 $\times$ 15 mm) to each of which is added 0.6 ml 72 % perchloric acid. With this amount of acid it is advisable to limit the area of paper to 6 cm^2 per tube.

The tubes are first heated at 100° for 1 min (this moderates the vigour of the subsequent oxidation) and then transferred to a metal block[4] which is maintained at 190° $\pm$ 2°, and into which the tubes fit to a depth of 4 cm.

To prevent water, which is formed in the oxidation, from condensing inside the tubes, it has been found convenient to surround the tubes with a cylindrical shield of aluminium foil which rests on the block and reaches to within 1 cm of the top of the tubes. If the liquid in the tubes is not free of colour after heating for 2 h, the rate of clearing can be accelerated by the addition of a drop of 30 % hydrogen peroxide, followed by further heating.

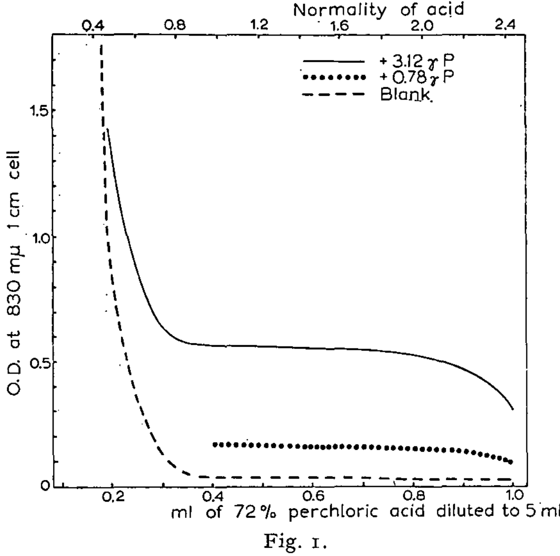

Fig. 1.

When the liquid is free of colour, the tubes are allowed to cool and the phosphate content of each is estimated as described by BARTLETT[1], a total volume of 5 ml being used during colour development.

Too high an acid concentration at this time will inhibit the formation of the blue colour, while insufficient acid gives rise to the production of considerable colour in tubes that contain no phosphorus. The useful concentration range for perchloric acid (determined in the absence of oxidised paper) was found to be from 0.9 *N* to 1.7 *N* (Fig. 1), and is similar to that found by BARTLETT for sulphuric acid[1]. BÖTTCHER *et al.*[4] recommend the use of 0.2 ml 70 % perchloric acid (equivalent to 0.48 *N* acid if diluted to 5 ml), but the total volume used during colour development is not stated.

The acid lost by the oxidation in the way described of 6 cm^2 Whatman No. 1 paper was found by titration to be equivalent to less than 0.05 ml 72 % perchloric acid. The final acid concentration is thus still within the recommended range.

Best results have been obtained using Whatman No. 1 paper which has been previously washed in 2 *N* acetic acid followed by distilled water and then dried[2]. Whatman No. 30 paper has also been found acceptable, but the phosphorus content of many untreated papers can be considerable.

The procedure described here has been applied largely to the determination of the rate of hydrolysis of phosphate diesters, and the analysis of the mixture of mono-esters so obtained.

Note added in proof

Certain mixtures of concentrated perchloric acid with organic materials have been known to explode on heating or mechanical shock[5]. Whilst no explosion has resulted during more than five hundred phosphorus determinations using this method, it is emphasised that due precautions should be taken during the heating stage (see also ref. 6).

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1 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
2 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
3 R. S. BANDURSKI AND B. AXELROD, *J. Biol. Chem.*, 193 (1951) 405.
4 C. J. F. BÖTTCHER, C. M. VAN GENT AND C. PRIES, *Anal. Chim. Acta*, 24 (1961) 203.
5 M. A. ELLIOT AND F. W. BROWN, *U.S. Bur. Mines, Rept. Invest.*, No. 4169 (1948) 17 pp.; C.A., 42 (1948) 2431 d.
6 R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 47.

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Identification of an artifact in the hydrolysis of choline-containing phospholipids

In the course of a study of the phospholipids of the South African pilchard (*Sardina ocellata* Jenyns), choline was liberated by hydrolysis of the material for 48 h in 6 *N* HCl at a temperature of 115° in a sealed tube. Choline was identified by chromatography on filter paper according to the method of LEVINE AND CHARGAFF[1,2]. Two blue spots were invariably obtained, one with the mobility of choline and the other a hitherto unidentified faster moving compound.

LEVINE AND CHARGAFF[1] hydrolyzed lecithin preparations and beef brain phosphatides with 6 *N* HCl at 100° for 48 h. These authors also described the appearance of two spots on paper chromatograms, when testing for choline with phosphomolybdic acid. They quoted an R_F value of 0.43 in a solvent system of *n*-butanol–diethylene glycol–water (4:1:1) for choline. However, for the unidentified substance in the above-mentioned solvent system, two R_F values were quoted: R_F = about 0.59 (purified lecithins) and $R_F = 0.53$ (beef brain phosphatides).

PHILLIPS[3] reported two compounds, which stained with phosphomolybdic acid, after hydrolysis of the lecithin and sphingomyelin fractions of human serum phospholipids with 6 *N* HCl at a temperature of 110° for 18 h. One compound, with an R_F value of 0.33 in the solvent system *n*-butanol–acetic acid–water (4:1:4, upper layer), was identified as choline; the other, with an R_F value of 0.41, remained unidentified.

LOVERN, OLLEY AND WATSON[4], in a study of the phospholipids of cod, hydrolyzed the phospholipids in a sealed tube with 2 *N* HCl at 120° for 24 h. These authors observed the presence of an unidentified compound occurring with choline in only one instance. It has been suggested that this unknown substance might be an unidentified nitrogenous base present in phospholipids[1,2]. The present paper shows that choline chloride when heated with aqueous HCl, gives rise to the formation of (2-chloroethyl)-trimethylammonium chloride. This compound is identical to the unidentified base obtained by hydrolysis of pilchard phospholipids with aqueous HCl.

Experimental

It was found in this laboratory that a solution of choline chloride in 6 *N* HCl heated for 48 h at 115° in a sealed tube and chromatographed on filter paper according to LEVINE AND CHARGAFF[1] produced two spots; one corresponding to choline and the other to the unidentified compound arising from the hydrolysis of pilchard phospholipids. Similarly, the hydrolytic procedures of LEVINE AND CHARGAFF[1], of PHILLIPS[3] and of LOVERN *et al.*[4] applied to choline chloride all gave rise to the formation of the unknown substance. This substance, therefore, appeared to be an artifact produced in the hydrolysis with HCl, and was subsequently identified as (2-chloroethyl)-trimethylammonium chloride. (2-Chloroethyl)-trimethylammonium chloride was synthesized according to TAKETOMO[5]*.

* Titratable chloride (argentometric): 22.36%; calculated for $C_5H_{13}NCl_2$ (158.07): 22.43%. No specific m.p. Decomposition at 249°. TAKETOMO[5] states decomposition at 243°, and TOLBERT[6] reports a m.p. of 300° with decomposition at 240–245°. The picrate of (2-chloroethyl)-trimethylammonium chloride had a m.p. of 212–214°. TOLBERT[6] found this to be 207°.

Chromatograms with choline chloride and (2-chloroethyl)-trimethylammonium chloride as marker compounds were run in different solvent systems, as shown in Table I, and no difference between the artifact produced on heating choline chloride with HCl, (2-chloroethyl)-trimethylammonium chloride and the unidentified compound in hydrolysates of pilchard phospholipids was observed. Mixtures of these compounds did not show any separation when chromatographed on filter paper.

TABLE I

R_F VALUES OF CHOLINE CHLORIDE AND (2-CHLOROETHYL)-TRIMETHYLAMMONIUM CHLORIDE IN DIFFERENT SOLVENT SYSTEMS ON WHATMAN NO. 1 PAPER

Solvent system	*R_F (20-24°)*				*Remarks*
	Choline chloride	*(2-Chloroethyl)-trimethyl-ammonium chloride*	*Artifact from choline chloride and HCl*	*Compound in hydrolysate of phospholipids*	
n-Butanol–diethylene glycol–water (4:1:1)	0.45	0.58	0.60	0.59	Descending
	0.40	0.55	0.54	—	Ascending
n-Butanol–acetic acid–water (4:1:4, upper layer)	0.31	0.39	—	0.39	Ascending
Ethanol–ammonia (95:5)*	0.48	0.56	0.58	—	Ascending
n-Butanol–pyridine (4:1) saturated with water	0.14	0.21	0.21	—	Ascending

* According to BREGOFF, ROBERTS AND DELWICHE[7].

An additional proof of the identity of the unknown substance was obtained by heating choline chloride in 6 *N* HCl at 115° for 168 h. Approximately 30 mg of the mixture was chromatographed on two sheets of filter paper (20 × 40 cm) with a descending solvent system *n*-butanol–diethylene glycol–water (4:1:1). The papers were dried in air and for 0.5 h at 100°. A guide strip was cut off the papers to locate the position of the faster moving compound, and the corresponding area containing this compound was cut out. The pieces of filter paper were first washed with ethyl ether to remove diethylene glycol, and thereafter extracted with 2 × 50 ml hot ethyl alcohol. After filtration the ethyl alcohol was removed *in vacuo*, and the remaining material treated with an aqueous solution of picric acid. The crystals obtained were filtered, washed with ethyl alcohol and dried over P_2O_5 *in vacuo*. The melting point of the crystals was 212–214°, and on mixing the material with the picrate of (2-chloroethyl)-trimethylammonium chloride no depression of melting point was observed.

The hydrolytic procedure of LOVERN *et al.*[4], *viz.* in 2 *N* HCl at 120° for 24 h, applied to choline chloride, produced only very small quantities of (2-chloroethyl)-trimethylammonium chloride, as judged by the intensity of the second spot on chromatograms. This method of hydrolysis did not produce (2-chloroethyl)-trimethylammonium chloride when applied to a pilchard phospholipid fraction, and was subsequently adopted in this laboratory. The hydrolysis according to LEVINE AND CHARGAFF[1] did not give as intense a second spot as the method of PHILLIPS[3]. Preference was, however, given to the method of LOVERN *et al.*[4].

Discussion

In the hydrolysis of lecithins[1], beef brain phosphatides[1,2], human serum phospholipids[3] and cod phospholipids[4] with aqueous HCl, an unidentified nitrogenous base, staining with phosphomolybdic acid has been found. This compound is in all probability (2-chloroethyl)-trimethylammonium chloride, and is formed by reaction of HCl with choline.

Hydrolysis of phospholipids with aqueous HCl therefore causes a loss of choline. The magnitude of the error introduced in quantitative choline determinations (in particular, LEVINE AND CHARGAFF's planimetric method[1]) was not investigated, but it appears from the intensity of the two spots produced that hydrolysis according to LOVERN *et al.*[4] and LEVINE AND CHARGAFF[1] only gives rise to the formation of small quantities of (2-chloroethyl)-trimethylammonium chloride.

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1 C. LEVINE AND E. CHARGAFF, *J. Biol. Chem.*, 192 (1951) 465.
2 C. LEVINE AND E. CHARGAFF, *J. Biol. Chem.*, 192 (1951) 481.
3 G. B. PHILLIPS, *Biochim. et Biophys. Acta*, 29 (1958) 594.
4 J. A. LOVERN, J. OLLEY AND H. A. WATSON, *J. Sci. Food Agr.*, 10 (1959) 327.
5 K. TAKETOMO, *C.A.*, 55 (1961) 9257d.
6 N. E. TOLBERT, *J. Biol. Chem.*, 235 (1960) 475.
7 H. M. BREGOFF, E. ROBERTS AND C. C. DELWICHE, *J. Biol. Chem.*, 205 (1953) 565.

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Modification of gas chromatographic columns by addition of glycerol

In this laboratory the estimation of volatile solvents normally occluded in colloidal propellants was attempted by means of gas chromatography. These solvents include methanol, ethanol, ethyl ether, acetone, ethyl acetate and water. The total content is usually about 1 %. Preliminary experiments with synthetic mixtures showed that water caused a serious interference, masking some of the constituents due to extreme "tailing".

The problem of "tailing" due to water has been encountered by many workers. The solution has generally been to use a highly polar liquid phase which retains the water until the organic constituents have been separated.

Several polar compounds, including polyethylene and polypropylene glycols supported on firebrick, in varying percentages were used in this laboratory for the

separation of the volatile matter in propellants. None of these compounds gave the desired results. Either the constituents were not separated or water interfered too seriously. It was found, however, that glycerol when added in small amounts to other liquid phases had a profound effect on the retention of water while only slightly affecting the emergence time of the other ingredients. In this respect it was found that incorporating 5 % of glycerol with 30 % of glycerol monoricinoleate on a firebrick support doubled the retention time of water. Increasing the glycerol content caused further retention of water. Although the interference due to water was eliminated all the other ingredients could not be separated with this modified column. When a small amount of glycerol was incorporated with 30 % tricresyl phosphate on a firebrick column all the volatiles were separated. Changes in retention times caused by the addition of glycerol to tricresyl phosphate (TCP) are shown in Table I. The apparatus used a cathetometer as detector. The columns were 0.25 in. in diameter, 12 ft. in length and maintained at 100°. The helium gas flow rate was 100 ml/min.

TABLE I

CHANGES IN RETENTION TIMES DUE TO ADDITION OF GLYCEROL

Compound	*Retention time (min)*	
	30 % TCP	*30 % TCP + 5 % glycerol*
Water	9.3	27.7
Methanol	8.0	10.0
Ethanol	11.6	15.2
Acetone	11.6	11.9
Ether	4.5	3.5
Ethyl acetate	19.0	16.8

The retentive effect on strongly polar compounds by the addition of glycerol to the chromatographic column is clearly evident. The modified column was able to separate all the ingredients while ethanol and acetone could not be separated with the one component liquid phase (30 % TCP). This technique is useful for the separation of low boiling polar and non-polar compounds and simplifies the choice of column materials since the screening of a large number of substrates is not necessary.

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Gas chromatography of digitoxigenin and digoxigenin*

Determination of the urinary excretion of digitalis compounds can improve dose control of digitalis in difficult clinical situations. FRIEDMAN, BINE, BYERS AND BLAND[1] have shown by bioassay that satisfactory complete digitalization is associated with a urinary digitoxin excretion of 40 μg per day, and that a urinary excretion of 80 μg per day is associated with imminent danger of toxicity. Unfortunately, their bioassay method is too laborious for routine clinical use.

Gas–liquid chromatography (GLC) has proven to be useful in the micro-analysis of steroids[2], sterols[3], bile acids[3,4], and sapogenins[5]. Since the aglycone moieties of digitalis compounds are steroids (Fig. 1), it was decided to investigate their behaviour when subjected to GLC.

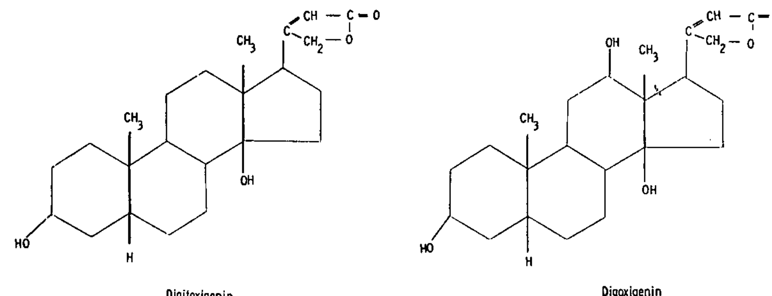

Fig. 1. Structures of digitoxigenin and digoxigenin.

Methods

A Barber Colman Model 10 gas chromatograph was used, employing 12 ft., 4 mm I.D. glass columns packed with 0.75 % SE-30 coated onto 100–140 mesh siliconized Gas Chrom P. A tritium argon ionization detector was used.

Trimethylsiloxy (TMS) derivatives of digitoxin, digoxin, digitoxigenin, and digoxigenin were made by the following reaction[6–8]:

$$2\ ROH + (CH_3)_3SiNHSi(CH_3)_3 \rightarrow 2\ ROSi(CH_3)_3 + NH_3.$$

1 mg of each reference compound was dissolved in 0.2 ml dry tetrahydrofuran and 0.4 ml hexamethyldisilazane was added. 0.1 ml trimethylchlorosilane was added to catalyze the reaction. The reaction mixture stood overnight at room temperature under a drying tube containing silica gel. The next morning any remaining liquid was evaporated with nitrogen. 2.0 ml of 30–60° petroleum ether was added, the residue triturated and centrifuged. The petroleum ether supernate was decanted, evaporated with nitrogen, and dissolved in 0.1 ml chloroform or light petroleum ether for GLC. The resulting structures of digitoxigenin-TMS and digoxigenin-TMS may be as shown in Fig. 2.

* Presented at the 35th Scientific Session American Heart Association, Cleveland, Ohio, Oct., 1962.

1 mg of each of the other compounds studied was dissolved in 0.1 ml chloroform or tetrahydrofuran for GLC*.

Results

Digitoxigenin-TMS and digoxigenin-TMS each gave single, separate, reproducible gaussian peaks on a column having 400 theoretical plates for cholestane (Fig. 3). Chromatographic conditions are given in the figure. Retention times were: cholestane 6.2 min; digitoxigenin-TMS 37.5 min; digoxigenin-TMS 47.5 min. The separation factor of digitoxigenin-TMS and digoxigenin-TMS was 1.26.

GLC of digitoxin, digoxin, and digoxigenin gave no useful peaks at all. GLC of

Digitoxigenin-TMS Digoxigenin-TMS

Fig. 2. Probable structures of digitoxigenin-TMS and digoxigenin-TMS.

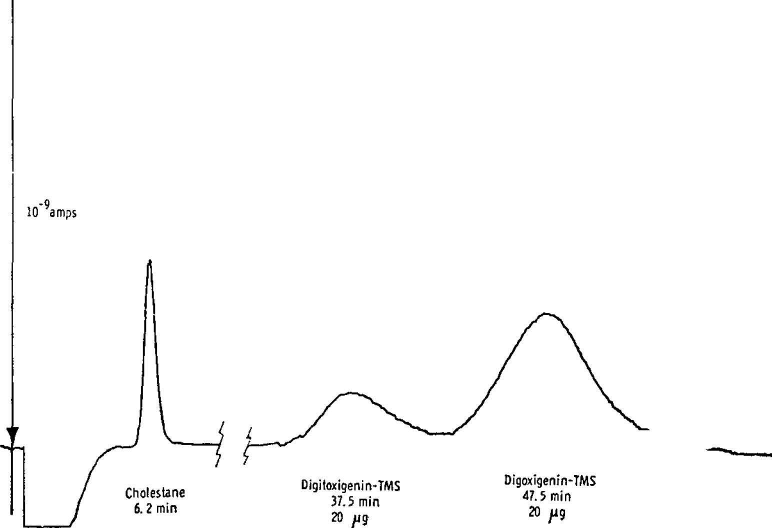

Fig. 3. Separation of digitoxigenin-TMS from digoxigenin-TMS by gas–liquid chromatography. A cholestane reference marker has been included in the mixture for chromatography. The chromatogram has been interrupted between cholestane and digitoxigenin-TMS to save space in the illustration. Column: 12 ft. 4 mm I.D. 0.75% SE-30, on 100–140 mesh Gas Chrom P. Column temp.: 228°. Detector temp.: 220°. Flash heater temp.: 348°. Detector volts: 1000. Inlet pressure: 40 lbs. Gas flow: 104 ml/min. Electrometer gain: 10^{-9} A.

* Digitoxigenin, digoxigenin, and digoxin were generously supplied by Burroughs-Wellcome and Company.

digitoxigenin gave a characteristic series of peaks which might have some use for identification purposes. These peaks were felt to represent pyrolysis products. For these studies, the column temperature was 249°, detector temperature 212°, flash heater temperature 307°, detector volts 1000, inlet pressure 60 p.s.i., and argon flow 130 ml/min.

Discussion

To our knowledge this represents the first separation of digitalis compounds by GLC. Amounts of TMS derivatives at present detectable permit the potential application of this method to urinary digitalis excretion studies. Hydrolysis of urinary glycosides to their aglycones may be required.

Potential determination of blood digitalis levels in patients on maintenance digitalis therapy will probably require at least a one-thousand fold increase in sensitivity. The bioassay method of FRIEDMAN AND BINE[9], which is sensitive to 0.05 μg/ml of serum, permits detection of digitalis in blood for 2 to 3 h following an intravenous full digitalizing dose of digitoxin, and cannot detect digitalis in the blood of patients on oral maintenance therapy. It seems desirable that a method for blood analysis be at least capable of detection of 0.01 μg in 10 to 20 ml of blood. An attempt to obtain this increased sensitivity by production of halogenated aglycones for GLC, using electron capture detection, is at present in progress.

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1 M. FRIEDMAN, R. BINE, JR., S. O. BYERS AND C. BLAND, *Circulation*, 2 (1950) 749.
2 W. J. A. VANDENHEUVEL, C. C. SWEELEY AND E. C. HORNING, *J. Am. Chem. Soc.*, 82 (1960) 3481.
3 W. L. HOLMES AND E. STACK, *Biochim. Biophys. Acta*, 56 (1962) 163.
4 J. SJÖVALL, C. R. MELONI AND D. A. TURNER, *J. Lipid Res.*, 2 (1961) 317.
5 W. J. A. VANDENHEUVEL AND E. C. HORNING, *J. Org. Chem.*, 26 (1961) 634.
6 T. LUUKKAINEN, W. J. A. VANDENHEUVEL, E. HAAHTI AND E. C. HORNING, *Biochim. Biophys. Acta*, 52 (1961) 599.
7 S. HASHMAN AND C. A. BROWN, *Inorg. Synth.*, 5, (1957). Chapter 4-A, Section 16, Part A.
8 S. H. LANGER, S. CONNELL AND I. WINDER, *J. Org. Chem.*, 23 (1958) 50.
9 M. FRIEDMAN AND R. BINE, JR., *J. Clin. Invest.*, 28 (1949) 32.

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Observations on the gas–liquid chromatography of n-monoalkenes with reference to the systematic identification of esters of unsaturated fatty acids through separation factors and log retention time plots

Introduction

Separation factors between any two materials in gas–liquid chromatography may be defined as the greater retention time divided by the lesser retention time. In the case of the methyl esters of unsaturated fatty acids on polyester substrates it has been possible to show that systematic relationships exist by separation factors among various acids of any one fixed chain length dependent on the relative positions of single double bonds or of normal methylene-interrupted multiple double bond systems[1,2]. These separation factors, designated type I and type II, are virtually independent of chain length and number of double bonds provided the differences in the number of double bonds are the same. It has been further suggested that these separation factors would also apply to related unsaturated materials, including hydrocarbons such as the n-alkenes.

In the type I and type II separation factors established for the methyl esters of unsaturated fatty acids it was possible, through varying the number of double bonds, to fix either the carboxyl end chain (the inclusive number of carbon atoms from the carboxyl group to the center of the first double bond) or the end carbon chain (the inclusive number of carbon atoms between the center of the last double bond and the terminal methyl group). In the type III separation factors the same end concepts are applied to chains of the same length containing the same number of double bonds in isomeric positions. The type I and type II relationships cannot be shown with the available hydrocarbon data, but the type III system can be explored, using the terms "long end carbon chain" and "short end carbon chain" to refer to the respective chain portions on either side of the center of the double bond in the n-monoalkenes. The effects of the different positions of the double bond systems for type I and II separation factors in the case of the longer chain fatty acids were ascribed to relative changes in volatility due to the three-carbon changes in the lengths of the carboxyl end chains and the end carbon chains. This was based on the observation that as the double bond in octadecenoic fatty acids approached either the carboxyl group[3], or particularly the terminal methyl group[2,4], of the fatty acid chain the retention time increased drastically. A similar observation may be made in part with octadecadienoic esters[5], while in the case of saturated esters of common chain length the same rule of increasing retention time is observed as either the acid or alcohol moiety of the chain is reduced[6]. In the latter example this may be compared with known boiling points of similar esters of shorter chain lengths[7], suggesting that the increased retention time is associated with a higher boiling point.

The separation factors to be examined are therefore of the type III system, with one double bond in various positions. The particular relationships are set up on the basis of the ratios of the "short end carbon chains" and are limited to pairs of

(Text continued p. 274)

TABLE I

A. COMPARISONS OF SOME SEPARATION FACTORS FOR *n*-ALKENES BASED ON 2/3, 3/1 AND 2/1 SHORT END CARBON CHAIN RATIOS

Column				I				II			
Reference				11				15			
Substrate				Silicone SF-96[a]				Hexadecane (with PrS and DmS)[b]			
Operating temperature				24°				25°			
Hydrocarbon	*B.P. °C*	*Long end carbon chain*	*Short end carbon chain*	*Rel. ret. time*[c]	*Separation factors* 2/3	3/1	2/1	*Rel. ret. time*[c]	*Separation factors* 2/3	3/1	2/1
cis-2-Heptene	98.41	5	2	3.19	—	—	—	—			
cis-3-Heptene	95.75	4	3	2.84	1.12	—	—	—			
1-Heptene	93.64	6	1	2.50	—	1.14	1.28	—			
cis-2-Hexene	68.89	4	2	1.16	—	—	—	1.42	—	—	—
cis-3-Hexene	66.45	3	3	1.03	1.12	—	—	1.26	1.12	—	—
1-Hexene	63.49	5	1	0.89	—	1.16	1.30	1.10	—	1.14	1.29
cis-2-Pentene	36.94	3	2	0.41	—	—	—	0.51	—	—	—
1-Pentene	29.97	4	1	0.31	—	—	1.32	0.39	—	—	1.31
trans-2-Heptene	97.95	5	2	2.95	—	—	—	—			
trans-3-Heptene	95.67	4	3	2.76	1.07	—	—	—			
1-Heptene	93.64	6	1	2.50	—	1.10	1.18	—			
trans-2-Hexene	67.88	4	2	1.05	—	—	—	1.31	—	—	—
trans-3-Hexene	67.09	3	3	1.03	1.02	—	—	1.21	1.08	—	—
1-Hexene	63.49	5	1	0.89	—	1.16	1.18	1.10	—	1.10	1.19
trans-2-Pentene	36.35	3	2	0.38	—	—	—	0.48	—	—	—
1-Pentene	29.97	4	1	0.31	—	—	1.22	0.39	—	—	1.23

B. COMPARISONS OF SOME SEPARATION FACTORS FOR n-ALKENES BASED ON 2/1 SHORT END CARBON CHAIN RATIOS

Column	*III*				*IV*				*V*			
Reference	8				8				8			
Substrate	*Silicone grease*[b]				*Silicone oil D.C. 200*[b]				*Polydiethylene glycol succinate*[b]			
Operating temperature	50°		100°		50°		100°		50°		100°	
Hydrocarbon	*Rel. ret. time*[c]	*Separation factor 2/1*	*Rel. ret. time*[c]	*Separation factor 2/1*	*Rel. ret. time*[c]	*Separation factor 2/1*	*Rel. ret. time*[c]	*Separation factor 2/1*	*Rel. ret. time*[c]	*Separation factor 2/1*	*Rel. ret. time*[c]	*Separation factor 2/1*
cis-2-Hexene	1.14		1.11		1.14		1.08		1.88		1.33	
1-Hexene	0.90	1.27	0.84	1.32	0.91	1.25	0.90	1.20	1.47	1.28	1.00	1.33
cis-2-Pentene	0.47		0.57		0.46		0.53		1.12		0.67	
1-Pentene	0.36	1.31	0.40	1.43	0.36	1.28	0.47	1.13	0.71	1.58	0.56	1.20
trans-2-Hexene	1.04		1.03		1.03		1.02		1.88		1.33	
1-Hexene	0.90	1.15	0.84	1.22	0.91	1.14	0.90	1.14	1.47	1.28	1.00	1.33
trans-2-Pentene	0.46		0.53		0.43		0.49		1.12		0.67	
1-Pentene	0.37	1.21	0.40	1.32	0.36	1.19	0.47	1.04	0.71	1.58	0.56	1.20

[a] Capillary column.
[b] Packed column.
[c] n-Hexane = 1.00 (B. P. 68.74°), all times adjusted.

alkenes with short end carbon chains of 1, 2 and 3 giving ratios of 2/3, 3/1 and 2/1. The appropriate separation factors will therefore be found under these headings in Table I.

Discussion

In the case of the gas–liquid chromatography of certain *n*-alkenes on a polyester substrate[8] (Table I-B, Column V) the 2/1 separation factors for the *cis*-hexenes and *cis*-pentenes are not the same, nor are the *trans* examples. Moreover the *cis* and *trans* forms of the same alkenes do not separate, indicating that on this polar substrate, although the retention times are in order of boiling point for the isomeric alkenes of the same chain length, there must be a further overriding influence, that of inductive forces from the respective end carbon chains, which would change the polarity of the double bonds in their interaction with the polyester. If the 2/1 separation factors for the butenes are also considered (1.43 at 50°, 1.33 at 100°), an alternating order of magnitude is apparent. The reversal of the relative magnitude of the separation factors for the pentenes with temperature increase further indicates that at moderate temperatures complex interactions govern the retention times of isomeric alkenes on polar substrates and hence the type III separation factors. However, as the temperature increases these same data indicate that the differences in the 2/1 separation factors for various chain lengths become less. Certainly the polarity of the 1-hexenes and 1-pentenes is notably altered, since they no longer separate from the corresponding alkanes at 100°. In the gas–liquid chromatography of the longer-chain unsaturated fatty acid esters with more centrally located unsaturation, inductive effects should play only a minor role in positional isomer separation at normal operating temperatures (180°–220°), although this would not apply to double bond systems very near the carboxyl group[9]. For example in the type II separation factors[2] it would be unreasonable to expect the inductive effects from the 3, 6 or 9 carbon atom end carbon chains to act universally on double bond systems containing from one to six double bonds.

A potential variable which has not been explored in alkene separations at moderate temperatures on polyester substrates is the physical state of the substrate. It has recently been shown[10] that the performance of inert solid and liquid hydrocarbon substrates in the gas–liquid chromatography of alkanes may differ drastically. Dependent on temperature, a polyester substrate might be solid, solid with a film of amorphous low molecular weight or degraded polyester, or a homogeneous liquid, with consequent variation in polarity and behaviour.

In contrast, examination of retention times at 24° for both *cis* and *trans* 2- and 3-alkenes in relation to 1-alkenes, on SF-96, an inert substrate[11] (Table I-A, Column I), not only gives very good correlation of retention times and boiling points, but also good agreement in the separation factors for the *cis*-heptenes and *cis*-hexenes. The *cis*-pentene data also agrees reasonably well, but the retention times for the shorter chain materials are not necessarily as satisfactory as for the longer chain materials[12]. Thus there is reason to believe (see also log plot discussion below) that the retention time for 1-pentene may be slightly low, and it will be noted that a relative retention time of 0.32 in lieu of 0.31 for this material would markedly improve the 2/1 separation factor correlation. This does not necessarily reflect on the accuracy of the experimental data, but rather on the number of significant figures in the particular relative retention time.

These observations suggest that in the case of the *cis* hydrocarbons on an "inert" substrate it may not be necessary to have the same "long end carbon chains" in order to have the same values for separation factors based on the ratios of the "short end carbon chains" only, provided that the number of carbon atoms in the "long end carbon chains" exceeds four or possibly three. This is in agreement with the type III separation factors obtained with polyunsaturated fatty acid esters on polyesters[2], where in those pairs of acids of common end carbon chain ratio the position of the unsaturation in relation to the carboxyl group is of little significance, provided the number of double bonds involved is the same in the various pairs of acids (*e.g.* 11,14- and 8,11-eicosadienoates and 9,12- and 6,9-octadecadienoates, end carbon chain ratios both 6/9, have respective type III separation factors of 1.03 and 1.04). Inductive forces are therefore unlikely to affect these separations. The particular acids in each set of pairs are those which may be respectively related by a linear log plot[13, 14].

Unfortunately the necessary data for longer chain hydrocarbons to support this view are not available, but some comparison may be made of data from different workers using the same shorter chain *cis* hydrocarbons on other "inert" substrates. At nearly the same temperature, data for a hexadecane column (coupled to a short section of column packed with *n*-propyl sulfone and 2,4-dimethylsulfolane)[15] agrees very well in the case of the *cis*-hexenes and *cis*-pentenes (Table I-A, Column II). At a somewhat higher temperature, 50°, silicone grease[8] also gives reasonably similar values, although these do not correlate at 100° (Table I-B, Column III). At the same elevated temperatures D.C. 200 silicone oil[8] (Table I-B, Column IV) gives very poor correlation. It will be noted that with the silicone grease both the *cis* and *trans* separation factors increase with increasing temperature, whereas with the silicone oil they decrease, suggesting that one of these last two substrates may not be truly non-polar (D.C. 200 silicone oil has been reported to be more polar than squalane[16]).

In the *trans* alkenes the situation is less clear, owing to the anomalous boiling point and retention time for the *trans*-3-hexene. The separation factors on hexadecane are interesting since the *trans*-hexene separation factors are virtually the same as those obtained for the *trans*-heptenes on SF-96. Unfortunately the former cannot be considered entirely reliable owing to the possible slight influence of the short length of mildly polar substrate preceding the hexadecane, although this does not seem to have an appreciable effect on the *cis* values, or on the *trans* 2/1 short end carbon chain ratio separation factor. In the case of the *trans* alkenes the silicone grease and D.C. 200 silicone oil separation factors are both in poor agreement with the SF-96 data.

In lieu of the common practice of plotting log retention time against boiling point, the log retention time data for the SF-96 column were plotted against number of carbon atoms[13, 14]. The C_4 points only partly fit the other data since the errors in relative retention time may be relatively large, but some significant correlations may be found. The *cis*-2-, *cis*-3- and *trans*-2-alkene lines are all parallel, and if the point for 1-pentene is slightly in error (see above), the 1-alkene line is also parallel to these. The *trans*-3-alkene line is abnormally inclined to the others owing to the anomalous *trans*-3-hexene point, and none are parallel to the *n*-alkane line. The latter is, however, based on data[11] at 25°. The results are therefore broadly similar to those suggested for positional isomers of methyl esters of certain polyunsaturated fatty acids on polyester substrates[13].

Conclusions

On limited evidence it appears that as operating temperatures increase, inductive effects in *n*-alkenes governing the polarity of the double bonds decrease, suggesting that other influences must govern the gas–liquid chromatographic separation of isomerically unsaturated materials on polar substrates at elevated temperatures.

The examination of hydrocarbon retention times on various substrates from the point of view of separation factors and in one case of the linear log plot system supports the viewpoint that these systematic relationships in the gas–liquid chromatography of unsaturated fatty acid esters on polyester substrates may be largely based on similarities in volatility due to contributions from similar structural elements.

The application of systematic separation factors to the analysis of hydrocarbon mixtures on strictly inert substrates may be useful if such mixtures are essentially straight-chain alkenes and if the relationships proposed can be shown to hold for longer chain materials where reference compounds of known structure may not be readily available.

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1 R. G. ACKMAN, *J. Am. Oil Chemists' Soc.*, submitted for publication.
2 R. G. ACKMAN AND R. D. BURGHER, *J. Chromatog.*, 11 (1963) 185.
3 A. T. JAMES, *J. Chromatog.*, 2 (1959) 552.
4 C. R. SCHOLFIELD, personal communication.
5 R. A. LANDOWNE AND S. R. LIPSKY, *Biochim. Biophys. Acta*, 46 (1961) 1.
6 D. LEFORT, C. PAQUOT AND A. POURCHEZ, *Oleagineux*, 16 (1961) 253.
7 K. S. MARKLEY, *in Fatty Acids, Their Chemistry and Physical Properties*, 1st. Ed., Interscience, New York, 1947, p. 169.
8 P. R. SCHOLLY AND N. BRENNER, in H. J. NOEBELS, R. F. WALL AND N. BRENNER (Editors), *Gas Chromatography*, Academic Press, New York, 1961, p. 263.
9 J. JANÁK, M. DOBIÁŠOVÁ AND K. VEREŠ, *Collection Czech. Chem. Commun.*, 25 (1960) 1566.
10 T. O. TIERNAN AND J. H. FUTRELL, *Anal. Chem.*, 34 (1962) 1838.
11 A. G. POLGÁR, J. J. HOLST AND S. GROENNINGS, *Anal. Chem.*, 34 (1962) 1226.
12 J. F. SMITH, *Nature*, 193 (1962) 679.
13 R. G. ACKMAN, *Nature*, 194 (1962) 970.
14 R. G. ACKMAN, *Nature*, 195 (1962) 1198.
15 M. G. BLOCH, in H. J. NOEBELS, R. F. WALL AND N. BRENNER (Editors), *Gas Chromatography*, Academic Press, New York, 1961, p. 133.
16 H. J. MAIER AND O. C. KARPATHY, *J. Chromatog.*, 8 (1962) 308.

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Book Review

Hochspannungselektrophorese—Ihre Anwendungsmöglichkeiten für biochemische und klinisch-chemische Trennprobleme, by R. CLOTTEN AND A. CLOTTEN, with a preface by Prof. Dr. Dr. h.c. Dr. h.c. L. HEILMEYER, Freiburg/Br, Georg Thieme Verlag, Stuttgart, 1962, xvi + 556 pages, 111 figs. including some colour plates, 113 tables, price cloth bound DM. 98.—.

The book under review was written by two workers who for the last few years worked on the task of transforming high voltage electrophoresis into a reliable clinical method for the analysis of non-protein body fluids.

A short general chapter reviews the physico-chemical principles of paper electrophoresis. This is followed by a longer chapter on the technique of high voltage electrophoresis including methods for desalting and deproteinising biological fluids and for the hydrolysis of proteins (about 100 pages). The next long chapter deals with colour reactions which may be used for the detection of separated compounds. Practically all classes of compounds are considered and detailed instructions for the preparation of reagents given (123 pages). An equally thorough and complete treatment is given of the quantitative methods for determining substances after separation (100 pages).

A final chapter entitled "Results" shows what separations can be obtained by high voltage electrophoresis and how they can be employed in clinical analysis. While the main emphasis is placed on body fluids and tissues in normal and pathological conditions, such topics as organic acids, alkaloids, nucleic acids, steroids, vitamins and even inorganic substances are equally well presented (134 pages). The book is an invaluable reference work not only in clinical chemistry and on high (and low) voltage electrophoresis but also for all chromatographers, as the chapters on colour reactions and quantitative determinations may be used equally well in chromatographic work and require no further reference to the literature. There are 111 illustrations some in colour and the reference list contains over 5000 references.

M. LEDERER (Rome)

Announcements

INTERNATIONAL SYMPOSIUM ON MICROCHEMICAL TECHNIQUES—1965

The Pennsylvania State University will conduct the International Symposium on Microchemical Techniques, August 22–27, 1965 at the Pennsylvania State University, University Park, Pa., U.S.A. The Symposium will be organized by The American Microchemical Society (formerly the Metropolitan Microchemical Society) and will be similar to the Symposium of 1961. The same committee is in charge. Details will be announced by the Society at a future date.

GAS CHROMATOGRAPHY SYMPOSIUM

The Fifth International Symposium on Gas Chromatography to be organized by the Institute of Petroleum Gas Chromatography Discussion Group will be held on September 8, 9 and 10, 1964 at Brighton, England. The main aim of the symposium will be to launch a concentrated attack on the underlying physical processes responsible for separation. To achieve this, the morning sessions will be entirely devoted to this theme, the subjects for the three morning sessions being "Techniques of Separation and Identification", "Column Design", and "Exploitation of Molecular Interactions". Each morning session will be commenced by two papers given by recognized authorities. Informal discussions will be held during the afternoon sessions on a wide range of subjects relevant to the development and application of the technique. As at previous symposia, the technical programme will be accompanied by a full social programme and an instrument exhibition. Intending delegates and those wishing to submit papers should write to:

The Organizing Office,
Fifth International Symposium on Gas Chromatography,
61 New Cavendish Street, London, W.1.

Bibliography Section

Paper Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

GREEN, J., MARCINKIEWICZ, S. AND MCHALE, D.: Paper chromatography and chemical structure. III. The correlation of complex and simple molecules. The calculation of R_M values for tocopherols, vitamins K, ubiquinones and ubichromenols from R_M(phenol). Effects of unsaturation and chain branching. *J. Chromatog.*, 10 (1963) 158–183 — 96 compounds studied.

MARCINKIEWICZ, S. AND GREEN, J.: Paper chromatography and chemical structure. IV. Intramolecular hydrogen bonding. *J. Chromatog.*, 10 (1963) 184–189 — 14 compounds studied.

3. TECHNIQUES I

DIRLIK, P. AND POSNER, H. S.: Automatic spraying of paper chromatograms. An apparatus based on rotary motion suitable for quantitative analysis. *J. Chromatog.*, 10 (1963) 250–251.

RANDERATH, K.: Chromatography of nucleotides on a new anion-exchange paper. *J. Chromatog.*, 10 (1963) 235 – 12 compounds chromatographed on polyethyleneimine paper.

STITCH, S. R. AND OAKEY, R. E.: The evaluation of radiochromatograms by liquid scintillation counting and photography. *Biochem. J.*, 81 (1961) 12P–13P.

4. TECHNIQUES II

Systematic analysis, automation and preparative scale paper chromatography

BANNARD, R. A. B. AND CASSELMAN, A. A.: Clam poison. II. Purification of clam poison residues of low toxicity by a heavy-paper technique. *Can. J. Chem.*, 39 (1961) 1879–1887; *C.A.*, 57 (1962) 9018e — preparative-scale paper chromatography.

6. ALCOHOLS

FREINKEL, N. AND DAWSON, R. M. C.: The synthesis of *meso*-inositol in germ-free rats and mice. *Biochem. J.*, 81 (1961) 250–254.

GIERER, J. AND NORÉN, I.: Über die Reaktionen des Lignins bei der Sulfatkochung. II. Modellversuche zur Spaltung von Aryl-alkyl-ätherverbindungen durch Alkali. *Acta Chem. Scand.*, 16 (1962) 1713–1729.

KISS, S. AND PETERFI, JR., S.: Paper-chromatographic method for identifying the phosphomonoesterases of the soil. *Studia Univ. Babes-Bolyai*, Ser. 2, No. 2 (1961) 292–296; *C.A.*, 57 (1962) 11582d — paper chromatography of glycerol liberated from Ca glycerophosphate.

PREUSS, F. R., WILLIG, G. AND FRIEBOLIN, H.: Zur Konfiguration renaler Ausscheidungsprodukte von 1-Äthinylcyclohexanol-(1) und 1-Äthinylcyclohexylcarbamat-(1). *Arch. Pharm.*, 296 (1963) 157–168.

SATO, T., FUKUYAMA, T., SUZUKI, T. AND YOSHIKAWA, H.: 1,2-Dihydro-1,2-dihydroxybenzene and several other substances in the metabolism of benzene. *J. Biochem.* (*Tokyo*), 53 (1963) 23–27.

7. PHENOLS

AKAGI, M. AND AOKI, I.: Food additives. VI. Metabolism of 2,6-di-*tert*-butyl-*p*-cresol in a rabbit. 1. Determination and paper chromatography of a metabolite. *Chem. Pharm. Bull.* (*Tokyo*), 10 (1962) 101–105; *C.A.*, 57 (1962) 10488a.

PENTTILÄ, A. AND SUNDMAN, J.: The structures of filixic acids. *Acta Chem. Scand.*, 17 (1963) 191–198.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

BIOL, H. AND MICHEL, A.: (Paper chromatography of coloured compounds of red wines). *Ann. Technol. Agr.*, 10 (1961) 312–324; *C.A.*, 57 (1962) 11671i.

BIOL, H. AND MICHEL, A.: (Chromatographic techniques for the differentiation of coloured materials of red wines). *Ann. Technol. Agr.*, 10 (1961) 325–337; *C.A.*, 57 (1962) 11672a.
BIOL, H. AND MICHEL, A.: (Chromatography of red wines derived from regulated wines). *Ann. Technol. Agr.*, 10 (1961) 339–344; *C.A.*, 57 (1962) 11672b.

9. OXO COMPOUNDS

GRYAZNOV, V. P. AND POLOZHENTSEVA, N. G.: (Spectrophotographic and chromatographic determination of carbonyl compounds in alcohol). *Tr. Tsentr. Nauchn.-Issled. Inst. Spirt. i Likero-Vodochn. Prom.*, No. 9 (1960) 84–88; *C.A.*, 57 (1962) 10356c.

10. CARBOHYDRATES

ASSARSSON, A. AND THEANDER, O.: Reaction of D-glucosone with methanol–hydrogen chloride. *Acta Chem. Scand.*, 17 (1963) 47–52.
AZUMA, I. AND YAMAMURA, Y.: Studies on the firmly bound lipids of human tubercle bacillus. II. Isolation of arabinose mycolate and identification of its chemical structures. *J. Biochem. (Tokyo)*, 53 (1963) 275–281.
BIDINOST, L. E.: Determination of dextran in wine by paper chromatography. *Anales Direc. Nacl. Quim. (Buenos Aires)*, 13 (1960) 39–41; *C.A.*, 57 (1962) 9010e.
IMAI, Y.: Biosynthesis of *myo*-inositol in the rat. *J. Biochem. (Tokyo)*, 53 (1963) 50–60.
IMANAGA, Y., SAITO, F. AND KUSUDA, Y.: Non-enzymic degradation of D-glucosaminic acid. *J. Biochem. (Tokyo)*, 53 (1963) 84–85.
KATSUSHIMA, N. AND HAKOMORI, S.: Abnormal polysaccharide–protein complex from an autopsy material of a novel familial metabolic disorder. *J. Biochem. (Tokyo)*, 53 (1963) 7–13.
MEIER, H.: Studies on a galactan from tension wood of beech (*Fagus silvatica* L.). *Acta Chem. Scand.*, 16 (1962) 2275–2283.
PANCZEL, M.: (Paper chromatographic tests on the sugar content of grapes). *Mitt. (Klosterneuberg), Ser. A, Rebe Wein*, 12 (1962) 124–130.
ROBERT, L. AND DISCHE, A.: Analysis of a sulfated sialofucoglucosaminogalactomannosidoglycan from corneal stroma. *Biochem. Biophys. Res. Commun.*, 10 (1963) 209–214.
SCHMIDT, G. H. AND BRUNNERT, H.: Quantitative Glykogen- und Zuckerbestimmung mit Hilfe der Papierchromatographie. *J. Chromatog.*, 10 (1963) 190–204 — quantitative analysis *in situ* (Ag).
STOEV, K. D., MAMAROV, P. T. AND BENCHEV, I. B.: (Chromatographic analysis of sugars and free amino acids during the upward and downward movement of sap in the grapewine). *Nauchni Tr. Vissh Selskostopanski Inst. "Georg Dimitrov" Agron. Fak. (Sofia)*, 7 (1959) 371–406; *C.A.* 57 (1962) 10228f.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BADAMI, R. C. AND GUNSTONE, F. D.: Vegetable oils. X. Component acids of *Argemone mexicana* seed oil by reversed-phase chromatography. *J. Sci. Food Agr.*, 13 (1962) 255–257; *C.A.*, 57 (1962) 11614i.
DAGLEY, S., TRUDGILL, P. W. AND CALLELY, A. G.: Synthesis of cell constituents from glycine by a *Pseudomonas*. *Biochem. J.*, 81 (1961) 623–631.
DANIELS, P. AND ENZELL, C.: Paper chromatography of resin acids. *Acta Chem. Scand.*, 16 (1962) 1530–1532 — glass fibre paper.
GENTILNI, L.: (Chromatography of salicylic acid in wines). *Staz. Sper. Viticolt. Enol. Ann. (Conegliano)*, 20, No. 12 (1960) 3; *C.A.*, 57 (1962) 10356h.
HARBORNE, J. B. AND CORNER, J. J.: Plant polyphenols. 4. Hydroxycinnamic acid-sugar derivatives. *Biochem. J.*, 81 (1961) 242–250.
HANSEN, H. J. M.: Quantitative paper chromatography of fatty acids. I. The influence of residual mobile phase. *Acta Chem. Scand.*, 17 (1963) 139–143.
HANSEN, H. J. M.: Quantitative paper chromatography of fatty acids. II. Some general considerations on direct photometry. *Acta Chem. Scand.*, 17 (1963) 187–190.
HORSCH, W. AND PROBST, H.: Bildung und Eigenschaften der 5-Hydroxy-5-äthyl-barbitursäure (Äthyldialursäure). *Arch. Pharm.*, 296 (1963) 249–256.
NAGAI, J.: Studies on itaconate metabolism. I. Itaconyl-CoA synthesizing reaction in cell-free extracts of *Pseudomonas fluorescens*. *J. Biochem. (Tokyo)*, 53 (1963) 181–187 — chromatography of hydroxamates.

12. ORGANIC PEROXIDES

OGATA, Y., FURUYA, Y., MAEKAWA, J. AND OKANO, K.: Kinetics of the acid-catalyzed transformation of peroxyacetic acid to acetyl peroxide in acetic acid. *J. Am. Chem. Soc.*, 85 (1963) 961–962.

13. STEROIDS

BOYD, G. S. AND MAWER, E. B.: The identification of Lifschütz-positive material appearing in the cholesterol fraction of lipid extracts. *Biochem. J.*, 81 (1961) 11P.

COHN, G. L. AND MULROW, P. J.: Androgen release and synthesis *in vitro* by human adult adrenal gland. *J. Clin. Invest.*, 42 (1963) 64–78.

CORREALE, L., BALESTRERI, R. AND ARCITI, C.: (Isolation of urinary 16-epiestriol by paper chromatography). *Arch. "E. Maragliano" Pathol. Clin.*, 18 (1962) 97–108; *C.A.*, 57 (1962) 10192i.

DANDA, J. AND HANOUSEK, J.: (Qualitative and quantitative determination of phytosterols in the unsaponifiable part of tall-oil resin by paper chromatography). *Papir Celulosa*, 17 (1962) 57–62; *Anal. Abstr.*, 10 (1963) 236.

FEHER, T., KOREF, O. AND HOLLO, I.: Paper chromatography of the neutral 17-keto steroids in normal human urine. *Acta Med. Acad. Sci. Hung.*, 18 (1962) 235–247; *C.A.*, 57 (1962) 12811b.

MARENZI, A. D. AND BRAEGGER, E. S.: (Chromatography of estrogens). *Rev. Asoc. Bioquim. Arg.*, 26 (1961) 45–48; *C.A.*, 57 (1962) 10131f.

OAKEY, R. E.: The separation of steroid oestrogens by partition chromatography. *Biochem. J.*, 81 (1961) 13P.

PASQUALINI, J. AND JAYLE, M. F.: Corticosteroid 21-sulphates in human urine. *Biochem. J.*, 81 (1961) 147–149.

SCHUBERT, K. AND HOBE, G.: (Chromatographic behaviour of phosphate esters of dehydroepiandroesterone (3β-hydroxy-androst-5-en-17-one) and testosterone). *Z. Physiol. Chem.*, 323 (1961) 264–269; *Anal. Abstr.*, 10 (1963) 313.

STERESCU, M. AND KAIN, N.: (Determination of testosterone phenylpropionate in the presence of testosterone). *Rev. Chim. (Bucharest)*, 13, No. 3 (1962) 172; *C.A.*, 57 (1962) 12812h.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

JONES, A. S. AND THOMPSON, T. W.: The detection of guanidine compounds on paper chromatograms. *J. Chromatog.*, 10 (1963) 248 — ninhydrin and alkali, U.V. fluorescence.

KOLÁŘ, B. AND KLÁCEL, Z.: (Impurities in technical Czechoslovak caprolactam). *Chem. Prumysl*, 12 (1962) 326–332; *Anal. Abstr.*, 10 (1963) 240.

MAYR, H. H. AND PAXTON, R. G.: Quaternary ammonium bases in the tomato plant. *Experientia*, 18 (1962) 440–441.

18. AMINO ACIDS

AVSHALUMOVA, A. D.: (Determination of meat freshness by paper chromatography). *Vopr. Pitaniya*, 21 (1962) 22–28; *C.A.*, 57 (1962) 8974f.

BOTVINIK, M. M. AND TROSHKO, E. V.: (Paper chromatography of amino acid esters and their detection as hydroxamates. II). *Zh. Obshch. Khim.*, 32 (1962) 1389–1390; *Anal. Abstr.*, 10 (1963) 296.

DOTTA, U.: (Qualitative and quantitative study of aminoaciduria in cattle by means of paper chromatography). *Atti Soc. Ital. Sci. Vet.*, 13 (1959) 161–165; *C.A.*, 57 (1962) 11704d.

FOWDEN, L.: A new asparagine derivative, N^4-(2-hydroxyethyl)-L-asparagine, from bryony (*Bryonia dioica*). *Biochem. J.*, 81 (1961) 154–156.

HATHAWAY, D. S. AND LIPSCOMB, H. S.: Chemical chromatographic and radioisotopic techniques in an experimental study of thyroid function. *Texas Rept. Biol. Med.*, 20 (1962) 204–213; *C.A.*, 57 (1962) 11772i.

JASKOSKI, J.: Paper chromatography of some fractions of *Ascaris suum* eggs. *Exptl. Parasitol.*, 12 (1962) 19–24; *C.A.*, 57 (1962) 10365d.

LACROIX, A. C. AND MONIER, J. C.: (Two-dimensional paper chromatography of serum and urine amino acids of French Algerian moslems with myoedema). *Bull. Soc. Pathol. Exotique*, 54 (1961) 947–951; *C.A.*, 57 (1962) 11748d.

LJUNGGREN, J. G.: The oxidation of 3,5-diiodotyrosine by peroxidase and hydrogen peroxide. *Acta Chem. Scand.*, 17 (1963) 567–572.

MATIKKALA, E. J. AND VIRTANEN, A. I.: A new γ-glutamylpeptide, γ-L-glutamyl-S-(prop-1-enyl)-L-cysteine, in the seeds of chives (*Allium schoenoprasum*). *Acta Chem. Scand.*, 16 (1962) 2461–2462.

MILDNER, P. AND ROBEK, V.: Quantitative determination of amino acids in the gliadin and glutelin of San Pastore wheat. *Kem. Ind. (Zagreb)*, 11 (1962) 227–233; *Anal. Abstr.*, 10 (1963) 404.

MUSZKATOWA, B.: (Studies on amino acid composition and protein supplementation in foods by use of paper chromatography). *Roczniki Panstwowego Zakladu Hig.*, 13 (1962) 151–160; *C.A.*, 57 (1962) 10295c.

NJAA, L. R.: Some problems related to detection of methionine sulphoxide in protein hydrolysates. *Acta Chem. Scand.*, 16 (1962) 1359–1362.

SAKAKIBARA, S., SHIN, K. H. AND HESS, G. P.: An approach to the specific cleavage of peptide bonds. I. The acyl migration in dipeptides containing hydroxyamino acids in anhydrous hydrogen fluoride. *J. Am. Chem. Soc.*, 84 (1962) 4921–4928 — retention analysis.

SCHILDKNECHT, H. AND BENONI, H.: Über die Chemie der Anziehung von Pollenschläuchen durch die Samenanlagen von Oenotheren. *Z. Naturforsch.*, 18b (1963) 45–54.

UBUKA, T.: Identification of isovalthine and its related compounds. *J. Biochem. (Tokyo)*, 52 (1962) 440–443.

WONG, F. F. AND CARSON, J. F.: Paper chromatography of some 2,4-dinitrophenyl S-alkyl-(L)-cysteines and corresponding sulfoxides. *J. Chromatog.*, 10 (1963) 242–245 — 6 plant amino acids.

YAMASHINA, I. AND IZUMI, K.: Chemistry of glycoproteins; the linkage between protein and polysaccharide. *Biochem. Med. Mucopolysaccharides*, (1962) 39–52; *C.A.*, 57 (1962) 8860g.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

HORISAKA, K. AND MUSASHI, A.: Über das aus Walmuskel isolierte Ophidin und Carnosin. *J. Biochem. (Tokyo)*, 53 (1963) 271–274.

JENSEN, K. B. AND VENNERØD, A. M.: A pharmacological and chromatographic comparison of the main kinin of human urine with bradykinin. *Acta Pharmacol. Toxicol.*, 19 (1962) 275–280.

KOTAKI, A.: Studies on insulin. V. On the structure of the glycyl chain of bonito insulin II. *J. Biochem. (Tokyo)*, 53 (1963) 61–70 — DNP-amino acids.

NARITA, K. AND ISHII, J.: N-terminal sequence in ovalbumin. *J. Biochem. (Tokyo)*, 52 (1962) 367–373

PHILLIPS, A. W. AND GIBBS, P. A.: Techniques for the fractionation of microbiologically active peptides derived from casein. *Biochem. J.*, 81 (1961) 551–556 — 100 peptide spots on finger prints.

SASAKAWA, S.: Studies on hemoglobin. VII. N- and C-terminal fragments derived from bovine globin α and β with N-bromosuccinimide oxidation. *J. Biochem. (Tokyo)*, 53 (1963) 188–200.

SATAKE, K. AND SASAKAWA, S.: Studies on hemoglobin. VIII. The tryptic peptides of bovine globin. *J. Biochem. (Tokyo)*, 53 (1963) 201–213 — finger printing.

SEU, J. H., SMEBY, R. R. AND BUMPUS, F. M.: Synthesis of alanyl^4-isoleucyl^5-angiotensin II. *J. Am. Chem. Soc.*, 84 (1962) 4948–4950 — solvent systems with acetonitrile and dimethylformamide.

SHEEHAN, J. C. AND McGREGOR, D. N.: Synthetic peptide models of enzyme active sites. I. Cyclo-glycyl-L-histidyl-L-serylglycyl-L-histidyl-L-seryl. *J. Am. Chem. Soc.*, 84 (1962) 3000–3005.

20. PROTEINS

FENTON, E. L.: The assay of insulin solutions by paper chromatography. *Biochem. J.*, 81 (1961) 570–575.

GOPPERS, V. AND PAULUS, H. J.: Macromolecular compounds isolated from airborne particles by electrophoresis and paper chromatography. *Am. Ind. Hyg. Assoc. J.*, 23 (1962) 181–185; *C.A.*, 57 (1962) 11486e.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BIANCHI, P. A., CRATHORN, A. R. AND SHOOTER, K. V.: The use of tritium-labelled thymidine in studies on the synthesis of deoxyribonucleic acids. *Tritium Phys. Biol. Sci., Proc. Symp. Detection Use, Vienna*, 2 (1961, Publ., 1962) 269–276; *C.A.*, 57 (1962) 10408i.

BLUMSON, N. L. AND BADDILEY, J.: Thymidine diphosphate mannose and thymidine diphosphate rhamnose in *Streptomyces griseus*. *Biochem. J.*, 81 (1961) 114–124.

HASHIMOTO, T. AND YOSHIKAVA, H.: Further studies on adenylyl 2,3-diphosphoglyceric acid. *J. Biochem. (Tokyo)*, 53 (1963) 219–226.

JONES, J. W. AND ROBINS R K.: Purine nucleosides. III. Methylation studies of certain naturally occurring purine nucleosides. *J. Am. Chem. Soc.*, 85 (1963) 193–201 — R_F values of 22 methylpurines and purine nucleosides in 8 solvent systems.

KOBATA, A.: The acid-soluble nucleotides of milk. II. Isolation and identification of two novel uridine nucleotide oligosaccharide conjugates from human milk and colostrum. *J. Biochem. (Tokyo)*, 53 (1963) 167–175.

LAPIDOT, Y. AND KHORANA, H. G.: The stepwise synthesis of ribo-oligonucleotides containing C_3'–C_5'-internucleotidic linkages. *J. Am. Chem. Soc.*, 85 (1963) 1363–1364.

RAMMLER, D. H. AND KHORANA, H. G.: Studies on polynucleotides. XVI. Specific synthesis of the C_3'–C_5' interribonucleotidic linkage. Examination of routes involving protected ribonucleosides and ribonucleoside-3'-phosphates Syntheses of uridylyl-(3' → 5')-adenosine, uridylyl-(3' → 5')-cytidine, adenylyl-(3' → 5')-adenosine and related compounds. *J. Am. Chem. Soc.*, 84 (1962) 3112–3122 — R_F values of 17 nucleotides.
SKORA, M.: (A paper chromatographic differentiation of some barbituric acid derivatives depending on degradation products). *Farm. Polska*, 17 (1961) 399–401; *C.A.*, 57 (1962) 11793b.
TSUMITA, T., IWANAGA, M. AND KOMAI, T.: Studies on deoxyribonucleic acids and nucleoproteins. II. Tritiation of deoxyribonucleic acids. *J. Biochem. (Tokyo)*, 52 (1962) 433–439.
WEIMANN, G. AND KHORANA, H. G.: Studies on polynucleotides. XVII. On the mechanism of internucleotide bond synthesis by the carbodiimide method. *J. Am. Chem. Soc.*, 84 (1962) 4329–4341 — R_F values of 30 various nucleotides and phosphates.

22. ALKALOIDS

HELLBERG, H.: On the photo-transformation of ergot alkaloids. IV. Lumilysergic amides. *Acta Chem. Scand.*, 16 (1962) 1363–1368.
KUHN, H. F. AND FRIEBEL, H.: Zum Nachweis des Codeins und seiner Metaboliten im Harn. *Arch. Pharm.*, 296 (1963) 232–235.
MARTINOD, P.: (Preliminary investigation of alkaloids from lupine by paper chromatography). *Ciencia Nat. (Quito)*, 5, No. 1 (1962) 3–6; *C.A.*, 57 (1962) 11554d.
STREET, H. V.: The rapid separation of drugs and poisons by high temperature reversed phase paper chromatography. 3. Alkaloids. *Acta Pharmacol. Toxicol.*, 19 (1962) 325–329 — R_F values of 31 alkaloids and related substances at 86° and 95° on tributyrin-impregnated paper.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

NASU, H. AND SAKAMOTO, Y.: Studies on the hydroxylation of the benzene moiety of the indole nucleus of indole compounds. III. On the enzymatic formation of 6-hydroxyindole-3-butyric acid. *J. Biochem. (Tokyo)*, 52 (1962) 351–357.
ROTH, H. J., NOUR EL DIN, H.: Zur Konstellation von Alkanolaminen. 2. Mitt. Umsetzung von Ephedra-Basen zu Dioxomorpholinen. *Arch. Pharm.*, 296 (1963) 265–270.
TABAK, S. V., GRANDBERG, I. I. AND KOST, A. N.: (Study of pyrazoles. XXV. Paper chromatography of pyrazolecarboxylic acids). *Zh. Obshch. Khim.*, 32 (1962) 1562–1564; *Anal. Abstr.*, 10 (1963) 207.

24. ORGANIC SULPHUR COMPOUNDS

STREET, H. V.: The rapid separation of drugs and poisons by high temperature reversed phase paper chromatography. 2. Phenothiazine tranquillizers and imipramine. *Acta Pharmacol. Toxicol.*, 19 (1962) 312–324 — R_F values of 9 phenothiazine derivatives at 86° and 95°.
UNO, T. AND UEDA, M.: Metabolism of sulfathiazole. II. Quantitative separation of the excrements in the human urine after administration of sulfathiazole. *Yakugaku Zasshi*, 82 (1962) 759–762; *C.A.*, 57 (1962) 9152f.
WAGNER, G. AND LENK, CH.: Thioglucoside von Mercaptophenylthioharnstoff und Mercaptophenylthiosemicarbazon-Verbindungen. *Arch. Pharm.*, 296 (1963) 281–295 — R_F values of 23 glucosides.

25. ORGANIC PHOSPHORUS COMPOUNDS

ÅGREN, G.: The isolation of phosphorylhomoserine from trichloroacetic acid extracts of *Lactobacillus casei*. *Acta Chem. Scand.*, 16 (1962) 1607–1615.
HACK, M. H. AND HELMY, F. M.: Lipid composition of human cerebrospinal fluid. *Proc. Soc. Exptl. Biol. Med.*, 111 (1962) 421–423.
ISHII, Y., HASHIMOTO, T., TATIBANA, M. AND YOSHIKAWA, H.: Erythronic acid 4-phosphate: an intermediate of inosine metabolism in human red cell hemolysates. *Biochem. Biophys. Res. Commun.*, 10 (1963) 19–22.
OHNOKI, S., SUZUE, G. AND TANAKA, S.: 5-Phosphomevalonic acid as the intermediate of the enzymatic synthesis of bacterial phytoene. *J. Biochem. (Tokyo)*, 52 (1962) 423–427.

27. VITAMINS

FRYDMAN, B. AND RAPOPORT, H.: Non-chlorophyllous pigments of *Chlorobium thiosulfatophilum*. Chlorobiumquinone. *J. Am. Chem. Soc.*, 85 (1963) 823–825.
MAHADEVAN, S. AND GANGULY, J.: Further studies on the absorption of vitamin A. *Biochem. J.*, 81 (1961) 53–58.

VIRTANEN, A. I. AND KIESVAARA, M.: On the purity of ascorbigen preparations and their antiscorbutic effect. *Acta Chem. Scand.*, 17 (1963) 848–849.

28. ANTIBIOTICS

JEFFERY, J. D. A., ABRAHAM, E. P. AND NEWTON, G. G. F.: Deacetylcephalosporin C. *Biochem. J.*, 81 (1961) 591–596.

YAKUBOV, G. Z., KHOKHLOVA, YU. M. AND BLINOV, N. O.: (Conditions for the paper chromatographic separation of the mycetin-violarin antibiotic group). *Mikrobiologiya*, 31 (1962) 526–533; *C.A.*, 57 (1962) 10131c.

ZAITSEVA, Z. M. AND ORLOVA, N. V.: (The physiological peculiarities of the *Streptomyces rimosus* mutant, LC-T-572, in the biosynthesis of oxytetracycline). *Mikrobiologiya*, 31 (1962) 449–453; *C.A.*, 57 (1962) 8996g.

29. INSECTICIDES AND OTHER PESTICIDES

SAMPAOLO, A.: (Search of metabolites of O,O-dimethyldithiophosphorylacetic acid N-methylamide in olive oil). *Ann. Chim. (Rome)*, 52 (1962) 319–331; *C.A.*, 57 (1962) 8914b.

SOKOLSKI, W. T., CHIDESTER, C. G. AND HONEYWELL, G. E.: The hydrolysis of methyl *p*-hydroxybenzoate by *Cladosporium resinae*. *Develop. Ind. Microbiol.*, 3 (1962) 179–187; *C.A.*, 57 (1962) 10352e.

30. SYNTHETIC AND NATURAL DYES

JENSEN, S. L.: Bacterial carotenoids. X. On the constitution of the minor carotenoids of *Rhodopseudomonas*. 1. P518. *Acta Chem. Scand.*, 17 (1963) 303–312 — R_F values of 6 carotenoids.

JENSEN, S. L.: Bacterial carotenoids. XI. On the constitution of the minor carotenoids of *Rhodopseudomonas*. 2. OH-R. *Acta Chem. Scand.*, 17 (1963) 489–499.

JENSEN, S. L.: Bacterial carotenoids. XII. The constitution of the minor carotenoids of *Rhodopseudomonas*. 3. OH-Y. *Acta Chem. Scand.*, 17 (1963) 500–508.

METCALF, R. L. AND NEWELL, I. M.: The biochromes of mites. *Ann. Entomol. Soc. Am.*, 55 (1962) 350–353; *C.A.*, 57 (1962) 10361h.

MISHUSTIN, E. N. AND NIKITIN, D. I.: (Susceptibility of humic acids to soil microflora). *Mikrobiologiya*, 30 (1961) 841–848; *C.A.*, 57 (1962) 10335g.

NASSHI, L.: (Radial chromatography of bile pigments). *Diagnosi Lab. Clin.*, 15 (1959) 145–162; *C.A.*, 57 (1962) 11469h — concentration of the sample for application.

PUCHE, R. C. T.: (Filter paper partition chromatographic analysis for certified, synthetic food colors). *Publ. Inst. Invest. Microquim., Univ. Nacl. Litoral (Rosario, Arg.)*, 25, No. 23–24 (1959–1960) 63–65; *C.A.*, 57 (1962) 10296h.

32. PHARMACEUTICAL APPLICATIONS

NOWELL, P. T., SCOTT, C. A. AND WILSON, A.: Determination of neostigmine and pyridostigmine in the urine of patients with myasthenia gravis. *Brit. J. Pharmacol.*, 18 (1962) 617–624; *C.A.*, 57 (1962) 9179d.

33. INORGANIC SUBSTANCES

ARNOLD, R. AND RITCHIE, J. F.: Ion-exchange paper chromatography of lanthanide ions. I. Anion exchange of lanthanide citrate complexes. *J. Chromatog.*, 10 (1963) 205–211.

BABKO, A. K., VDOVENKO, M. E. AND KOPA, M. V.: (Direct photometric estimation of rare earths after paper chromatographic separation). *Zavodsk. Lab.*, 29 (1963) 645–649 — paper impregnated with NH_4NO_3; mobile phase ether–acetone–HCNS.

BISWAS, S. D. AND DEY, K. A.: A scheme of separation of common inorganic cations and identification by filter paper strip chromatography. *Z. Anal. Chem.*, 192 (1963) 376–378.

DOLCINI, C. AND DOLCINI, B. C.: (Micro-determination of silicon in tissues and in biological materials — chromatographic technique for the separation of silicon and phosphorus, respectively, as silicate and phosphate). *Riv. Biol. (Perugia)*, 52 (1960) 199–217; *C.A.*, 57 (1962) 12812i.

GROSS, D.: An unusual ninhydrin reaction with inorganic cations. *J. Chromatog.*, 10 (1963) 233–234.

34. RADIOACTIVE COMPOUNDS

EHRENBERG, A., EHRENBERG, L. AND LÖFROTH, G.: Degradation products of γ-irradiated solid carbohydrates obtained after dissolution in water. *Acta Chem. Scand.*, 17 (1963) 53–56.

TARCHEVSKIĬ, I. A. AND KARPILOV, YU. S.: (On the nature of the products of short time photosynthesis). *Fiziol. Rast.*, 10 (1963) 229–231.
TOWNE, J. C. AND GATTI, B. M.: Demonstration of radioautography and paper chromatography using a polaroid camera. *J. Chem. Educ.*, 40 (1963) 243–244.
VESTER, F. AND REITER, W.: Identifizierung von Substanzen durch Simultan-Chromatographie isotopen-markierter Verbindungen. *Experientia*, 18 (1962) 385–387.

Thin-Layer Chromatography

1. REVIEWS AND BOOKS

TRUTER, E. V.: *Thin Film Chromatography*. Cleaver-Hume Press, London, 1963, 205 pp.

2. FUNDAMENTALS, THEORY AND GENERAL

GEISS, F. AND SCHLITT, H.: Der Einfluss der relativen Feuchte bei der Vorbehandlung und Entwicklung von Dünnschicht-chromatographie-platten. *Naturwissenschaften*, 50 (1963) 350–351.
KELEMEN, J. AND PATAKI, G.: Einfluss der Schichtbereitung auf die Reproduzierbarkeit der R_F-Werte in der Dünnschichtchromatographie. II. Vergleich luftgetrockneter und aktivierter Schichten. *Z. Anal. Chem.*, 195 (1963) 81–86.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

LAMBERTSEN, G. AND HOLMAN, R. T.: Partial characterization of the hydrocarbons of herring oil. *Acta Chem. Scand.*, 17 (1963) 281–282.

10. CARBOHYDRATES

BERGEL'SON, L. D., DYATLOVITSKAYA, E. V. AND VORONKOVA, V. V.: (Descending thin-layer chromatography of polyhydroxy compounds). *Dokl. Akad. Nauk SSSR*, 149 (1963) 1319–1321 — cellulose layers.
WASSERMAN, L. AND HANUS, H.: Dünnschichtchromatographie von Zuckern und Zuckeralkoholen. *Naturwissenschaften*, 50 (1963) 351 — mixture of 60% kieselgur with 40% silica gel; R_F values of 12 sugars.
ZHDANOV, YU. A., DOROFEENKO, G. N. AND ZELENSKAYA, S. V.: (Thin-layer chromatography of carbohydrates on gypsum). *Dokl. Akad. Nauk SSSR*, 149 (1963) 1332–1333 — R_F values of 16 sugars.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

OKUI, S., UCHIYAMA, M. AND MIZUGAKI, M.: Metabolism of hydroxy fatty acids. I. Metabolic conversion of ricinoleic acid by a certain microorganism to 8-D-(+)-hydroxy-tetradec-*cis*-5-enoic acid. *J. Biochem. (Tokyo)*, 53 (1963) 265–270 — detection in ultra-violet light (365 mμ) after spraying with 2',7'-dichlorofluorescein.
STOFFYN, P. AND STOFFYN, A.: Direct conversion of sulfatides into cerebrosides. *Biochim. Biophys. Acta*, 70 (1963) 107–108.
YAMAKAWA, T., YOKOYAMA, S. AND HANDA, N. (née KISO): Chemistry of lipids of posthemolytic residue or stroma of erythrocytes. XI. Structure of globoside, the main mucolipid of human erythrocytes. *J. Biochem. (Tokyo)*, 53 (1963) 28–36 — Thin-layer chromatography of globoside and paper chromatography of sugar components.

13. STEROIDS

DUMAZERT, CH., GHIGLIONE, C. AND PUGNET, T.: Sur la chromatographie en couches minces de quelques stéroïdes. *Ann. Pharm. Franc.*, 21 (1963) 227–232 — a mixture of silica gel with alumina.
HAAHTI, E. AND NIKKARI, T.: Separation and isolation of waxes and sterol esters of skin surface fat with thin layer chromatography. *Acta Chem. Scand.*, 17 (1963) 536–537.
HAAHTI, E., NIKKARI, T. AND JUVA, K.: Fractionation of serum and skin waxes with chromatography on silica gel impregnated with silver nitrate. *Acta Chem. Scand.*, 17 (1963) 538–540.
HOFMANN, A. F.: The preparation of chenodeoxycholic acid and its glycine and taurine conjugates *Acta Chem. Scand.*, 17 (1963) 173–186 — R_F values of 9 steroid acids.

15. TERPENE DERIVATIVES

MOUTON, M., JAQUARD, S. AND SAGOT-MASSON, M.: Séparation de l'acide asiatique par chromatographie en couches minces sur poudre de verre. Dosage de l'acide asiatique sur un éluat. *Ann. Pharm. Franc.*, 21 (1963) 233–238.

STEIN, G.: Über den Sexuallockstoff von Hummelmännchen. *Naturwissenschaften*, 50 (1963) 305.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

KNABE, J. AND KRÄUTER, R.: Die aromatischen Reaktionsprodukte der alkalischen Hydrolyse des Chloramphenicols. *Arch. Pharm.*, 296 (1963) 190–205 — azo and nitro compounds.

18. AMINO ACIDS

CALLAHAN, F. M., ANDERSON, G. W., PAUL, R. AND ZIMMERMAN, J. E.: The tertiary butyl group as a blocking agent for hydroxyl, sulfhydryl and amido functions in peptide synthesis. *J. Am. Chem. Soc.*, 85 (1963) 201–207 — thin-layer and paper chromatography of amino acid derivatives.

22. ALKALOIDS

GRÖGER, D. AND ERGE, D.: Dünnschichtchromatographie von Mutterkornalkaloiden. *Pharmazie*, 18 (1963) 346–349 — R_F values of 12 water-soluble ergot alkaloids developed on silica gel, kieselguhr and alumina.

MOLL, F.: Dünnschichtchromatographie von Schierlingsalkaloiden und anderen Piperidinbasen. *Arch. Pharm.*, 296 (1963) 205–207.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

JENSEN J.: Separation of the coproporphyrin isomers I and III by thin-layer chromatography. *J. Chromatog.*, 10 (1963) 236–238.

24. ORGANIC SULPHUR COMPOUNDS

EIDEN, F. AND STACHEL, H. D.: Das chromatographische Verhalten von Phenothiazin-Derivaten an verschiedenen Adsorbentien. *Deut. Apotheker-Ztg.*, 103 (1963) 121 — R_F values of 14 phenothiazine derivatives; comparison of thin-layer chromatography with the results obtained on paper containing inorganic adsorbents.

WHITE, E. H., MCCAPRA, F. AND FIELD, G. F.: The structure and synthesis of firefly luciferin. *J. Am. Chem. Soc.*, 85 (1963) 337–343 — thin-layer and paper chromatography.

25. ORGANIC PHOSPHORUS COMPOUNDS

KLEMENT, R. AND WILD, A.: Dünnschichtchromatographie von Phosphorsäureestern. *Z. Anal. Chem.*, 195 (1963) 180–185 — R_F values of 14 compounds on silica gel.

STEIN, Y., STEIN, O. AND SHAPIRO, B.: Enzymic pathways of glyceride and phospholipid synthesis in aortic homogenates. *Biochim. Biophys. Acta*, 70 (1963) 33–42.

SVENNERHOLM, L.: Isolation of gangliosides. *Acta Chem. Scand.*, 17 (1963) 239–250 — thin-layer and paper chromatography.

SVENNERHOLM, L.: Isolation of the major ganglioside of human spleen. *Acta Chem. Scand.*, 17 (1963) 860–862.

27. VITAMINS

HASSELQUIST, H. AND JAARMA, M.: Dünnschicht-chromatographische Bestimmung der Askorbinsäure in Kartoffelknollen. *Acta Chem. Scand.*, 17 (1963) 529–533.

32. PHARMACEUTICAL APPLICATIONS

BÄUMLER, J. AND RIPPSTEIN, S.: Über den Nachweis von Cabromal (Adalin) und dessen Metaboliten. *Arch. Pharm.*, 296 (1963) 301–306.

34. RADIOACTIVE COMPOUNDS

KARLSON, P., MAURER, R. AND WENZEL, M.: Eine Mikromethode zur Markierung von Steroiden und von Ecdyson mit Tritium. *Z. Naturforsch.*, 18b (1963) 219–224 — pregnane derivatives.

Gas Chromatography

1. REVIEWS AND BOOKS

HARDY, C. J.: Gas chromatography. *Nature*, 198 (1963) 741–743 — technical review of the General Annual Meeting of the Gas Chromatography Discussion Group, London, April 5, 1963.

2. FUNDAMENTALS, THEORY AND GENERAL

2c. Thermodynamics and relations

DHONT, J. H.: Comparison of relative retention-time data and the Kovats index. *Nature*, 198 (1963) 990–991 — indexes are superior to the relative retention times.

2d. General

GIRAUD, A.: (Gas chromatography: quantitative determinations). *Rev. Inst. Franc. Petrole Ann. Combust. Liquides*, 18 (1963) 271–283 — methodics.

HEWITT, G. F. AND SHARRATT, E. W.: Gaseous diffusion in porous media with particular reference to graphite. *Nature*, 198 (1963) 952–957 — a critical review (45 references) from the theoretical point of view; well suited to application in GC.

JOHNS, T.: Selective detection and identification of pesticide residues. *Abstr. 3A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — selectivity is achieved by means of an electric conductivity component incorporated into the flame ionization detector.

3. TECHNIQUES I

3a. Detectors

KLAVER, R. F., AND LETOURNEAU, R. L.: A fast mass spectrum digitizer for a gas chromatograph–mass spectrometer combination. *28th Midyear Meeting Am. Petroleum Inst. Div. Refining, Philadelphia, Pa., May 13–16, 1963.*

MIDDLEHURST, J. AND KENNETT, B.: Flame ionization detectors. *J. Chromatog.*, 10 (1963) 294–302 — details of flame ionization triode detector. Linearity $1:10^8$; detection limit 10^{-13} g/sec.

PERKINS, JR., G., KANG YANG AND FOLMER, JR., O. F.: Measurement of ion formation during combustion. *Nature*, 198 (1963) 780–781 — the kinetics of combustion reactions can be studied on catalysts better than in flames.

SCHMAUCH, L. J.: Thermal conductivity cell apparatus. *U.S. Pat.*, 3,075,379 (Jan. 29, 1963) — matched resistored Wheatstone bridge.

3b. Column performance and filling studies

BROWN, I.: The role of the stationary phase in gas chromatography. *J. Chromatog.*, 10 (1963) 284–293 — classification is based on triangular graphs of retention values for three electron donors or acceptors.

HAWKES, S. J.: The effect of stationary phase viscosity on column efficiency. *GC Discussion Group Meeting, London, April 5, 1963*; from *Nature*, 198 (1963) 743 — diffusivity term should be almost proportional to 1/viscosity.

HELLYER, R. O. AND KEYZER, H.: The effect of pressure differentials on gas chromatography separation. *J. Chromatog.*, 10 (1963) 314–323 — separation of components from mixtures by means of three parallel columns and pressure programming.

HUBER, J. F. K. AND KEULEMANS, A. I. M.: Modifications of solid supports for the gas liquid chromatographic measurement of the characteristic of solid supports. *GC Discussion Group Meeting, London, April 5, 1963*; from *Nature*, 198 (1963) 742–743 — heating of Sterchamol for 100 h to 1150° reduced the specific surface without noticeably changing the specific volume.

MORTIMER, J. V. AND GENT, P. L.: The use of organo-clays as stationary phases. *GC Discussion Group Meeting, London, April 5, 1963*; from *Nature*, 198 (1963) 742 — separation of *p*- and *m*-xylenes on Bentone 34 at 70°.

POPE, C. G.: Gas solid elution chromatography with graphitized carbon black. *Anal. Chem.*, 35 (1963) 654–658 — carbon black with 89.4 m^2/g (B.E.T.) $<$ 170 mesh coated on polythene molding powder (100–120 mesh) at 160–170°, 1:1. C_6–C_7 symmetrical at 110°.

SCOTT, R. P. W.: Preparative scale chromatography with analytical columns. *Nature*, 198 (1963) 782–783 — 1 ml liquid/1 cm I.D. column gives symmetrical peaks and good resolution if temperature programming takes place.

3c. Apparatus, accessories and materials for GC

BURNELL, M. R. AND STERNBERG, J. C.: Far-out chromatography. *Abstr. 18-J, Div. Ind. Engng. Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — lunar chromatograph for studying the atmosphere, and the pyrolysis of solid earth samples.

CAVE, G. C. B.: The collection and analysis of odorous gases from Kraft pulp mills. Part IV. A field kit for the collection of the pollutants and methods for their analysis. *Tappi*, 46 (1963) 15–20.

CLARKE, D. R.: A quantitative gas stream splitting injection system suitable for use with capillary columns. *Nature*, 198 (1963) 681–682.

KARMEN, A., MCCAFFREY, I. AND BOWMAN, R. L.: The validity of the results obtained when a stream-splitting device is used before the gas–liquid chromatography detector. *J. Lipid Res.* 4 (1963) 108–110 — splitting to mass detector and for radioactive measurement on anthracene crystal; separation of fatty acids methyl esters.

REINECKE, M. E.: Shut off valve for chromatographic systems. *U.S. Pat.*, 3,077,766 (Febr. 19, 1963).

SHEARER, D. A. AND STONE, B. C.: Collection of gas-chromatographic fractions for identification by infrared spectrophotometry. *Analyst*, 88 (1963) 147–149 — for samples of 0.2 mg; detailed scheme.

STEVENS, R. K. AND MOLD, J. D.: A gas chromatographic trap designed to collect compounds which tend to form aerosols. *J. Chromatog.*, 10 (1963) 389–399 — detailed design.

4. TECHNIQUES II

4a. Preparative scale GC

GOLAY, M. J. E.: Some implications of recirculated gas chromatographic columns. *GC Discussion Group Meeting, London, April 5, 1963*; from *Nature*, 198 (1963) 741 — recirculated GC is an economic method in g to kg scale; resolution is proportional to n only.

JENTOFT, R. E., JOHNSON, J. F. AND CARLSTROM, A. A.: Preparative gas chromatography. *Abstr. 17-J, Div. Ind. Engng. Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — recirculated GC.

ROSE, A. AND SUMANTRI, R. B.: Rigorous calculations for prediction of scaled-up gas chromatography. *Abstr. 18-J, Div. Ind. Engng. Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

SCOTT, R. P. W.: The heavy-loading of analytical scale column. *GC Discussion Group Meeting, London, April 5, 1963*; from *Nature*, 198 (1963) 741–742 — symmetrical peaks obtainable by interruption of the gas flow for a certain time and by increasing the flow rate exponent.

4d. Special microtechniques

KALLEND, A. S. AND PITTS, JR., J. N.: Application of high sensitivity, repeated sampling, gas chromatographic technique to studies of photochemical systems. *Abstr. 25-R, Div. Water Waste Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

LEVI, L., NIGAM, I. C. AND DAVIES, L.: Essential oils analysis by coupled gas-liquid thin-layer chromatography. *Abstr. 18-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

SETZKORN, E. A. AND CAREL, A. B.: The analysis of alkyl aryl sulfonates by micro desulfonation and gas chromatography. *J. Am. Oil Chemists' Soc.*, 40, No. 2 (1963) 57–59.

4e. Automation

ARUTYUNOV, YU. I., FRID, M. N., BRESHCHENKO, V. YA., PINCHEVSKAYA, S. I. AND FRID, E. B.: (Chromatothermographic analysis of feed and pyrolysis product streams). *Khim. Tekhnol. Topl. Masel*, No. 3 (1963) 43–47 — experience with GC XT-2M for C_1–C_5 hydrocarbons.

HEATON, WM. B.: Chromatographic method and apparatus. *U.S. Pat.*, 3,077,103 (Febr. 12, 1963) — annular chromatographic column formed from cocentric coaxial cylindrical members.

REED, D. W.: New portable analyzer solves process problems. *Oil Gas J.*, 61 (1963) 68–69 — portable automatic apparatus for Class 1, Division 2 area.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

EDMONDS, S. M., TURK, A., MORROW, J., DILL, A. AND DAVIS, S.: Determination of the efficiency of brominated activated charcoal (BAC) in removing light olefins from a gas stream. *Abstr. 29-R, Div. Water Waste Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

JEUNG, E. AND HELWIG, H. L.: Method for analysis of C_1–C_4 hydrocarbons in atmospheric samples by gas chromatography. *Abstr. 26-R, Div. Water Waste Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* – 1 ml air; detection limit 1.40 p.p.m. CH_4–0.01 p.p.m. C_4.

ZORIN, A. D., ESHELEVA, A. E. AND DEVYATYKH, G. G.: (Estimation of solution ability of gases by means of gas–liquid partition chromatography). *Zavodsk. Lab.*, 29 (1963) 659–662 — values for CO_2, C_1–C_4 hydrocarbons in nitrobenzene.

5b. Other hydrocarbon types

DURRETT, L. R., TAYLOR, L. M., WANTLAND, C. F. AND DVORETZKY, I.: Component analysis of isoparaffin-olefin alkylate by capillary gas chromatography. *Anal. Chem.*, 35 (1963) 637–641 — C_3–C_{12} alkylate on squalane in 200 ft., 0.010 in. stainless steel tubes, at 26–100°; splitter 1:50–1000 at 300–350°.

EYZEN, O. G., RANG, S. A. AND ARUMEEL, E. KH.: (About the chemical composition of paraffin-naphthenic part of 150–215° cut of tar). *Khim. Tekhnol. Topl. Masel*, No. 5 (1963) 38–42 — quantitative analysis of C_6–C_9 aromatics.

HAUSER, C. F., BROOKS, T. W., MILES, M. I., RAYMOND, M. A. AND BUTLER, G. B.: The Wittig synthesis. I. Use of certain aliphatic aldehydes as intermediates in the synthesis of olefins. *J. Org. Chem.*, 28 (1963) 372–379 — olefins on Carbowax 1000 and diethylene glycol succinate polyester.

MILVITSKAYA, E. M. AND PLATÉ, A. F.: (Isomerization in the presence of silica gel of some bi- and tricyclic hydrocarbons containing a three-membered ring). *Neftekhimiya*, 3 (1963) 188–197.

MINACHEV, KH. M. AND LOGINOV, G. A.: (Conversions of alkyl cyclopentenes and of allyl cyclopentane on erbium oxide). *Neftekhimiya*, 3 (1963) 181–187 — $AgNO_3$ in ethylene glycol.

NELSON, R. L. AND BERG, L.: Azeotropic separation of ethylbenzene from *p*-xylene. *Abstr. 4-O, Div. Petroleum Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

POLYAKOVA, T. A., SOKOLOVA, T. A. AND PARFIN, YA. A.: (Analysis of catalytic hydrogenation products of furan by means of gas–liquid chromatography). *Zavodsk. Lab.*, 29 (1963) 664–665 — 68°; tricresyl phosphate.

SAUERLAND, H. D.: (New developments in gas chromatography of coal tar products). *Brennstoff-Chem.*, 44/2 (1963) 37–43 — programmed separation of compounds above anthracene; SE-30, 20% Chromosorb 30/60; 1.25 m; 90°/250°: 5°/min.

SREENIVASAN, B., NOWAKOWSKA, J., JONES, E. P., SELKE, E. SCHOLFIELD, C. R. AND DUTTON, H. J.: Hydrogenation of linolenate. VII. Separation and identification of isomeric dienes and monoenes. *J. Am. Oil Chemists' Soc.*, 40, No. 2 (1963) 45–51 — separation of *cis-trans* and *trans-cis* derivatives; 150–200°; polyethylene glycol succinate polyester, Apiezon L.

WEISS, J., COLLINS, C., CARCIELLO, N. AND SUCHER, J.: Radiolytic and pyrolytic stability of some polyaromatic compounds. *Abstr. 5-J, Div. Ind. Engng. Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — programmed GC and flame ionization detector; aromatic hydrocarbons b.p. 200–450°.

5c. Halogen derivatives of hydrocarbons

BAKER, F. W., HOLTZ, H. D. AND STOCK, L. M.: The brominative decarboxylation of bicyclo-[2,2,2]-octane-1-carboxylic acid in halogenated solvents. *J. Org. Chem.*, 28 (1963) 514–516 — halobicyclic-[2,2,2]-octanes on Dow Corning high vacuum grease at 100°.

BELLAR, T. A. AND SIGSBY, J. E.: Application of electron capture detector to gas chromatography in air pollution. *Abstr. 25-R, Div. Water Waste Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

PURCHASE, I. F. H.: Estimation of Halothane tensions in blood by gas chromatography. *Nature*, 198 (1963) 895–896 — error $\pm$ 2.5%.

6. ALCOHOLS

DAVIS, T. W. M., FARMILO, C. G. AND OSADCHUK, M.: Identification and origin determinations of *Cannabis* by gas and paper chromatography. *Anal. Chem.*, 35 (1963) 751–755 — composition of cannabinol and diol derivatives on SE-30 at 212° varies in accordance with geographic origin.

FEDOROVA, V. V., PAVLOV, G. P. AND SINOVICH, I. D.: (Preparation of hexanetricol-1,2,6 from acrolein). *Neftekhimiya*, 3 (1963) 259–266.

9. OXO COMPOUNDS

RAMSHAW, E. H.: Gas chromatography of vinyl ketones. *J. Chromatog.*, 10 (1963) 303–308 — retention data for C_3–C_{12} saturated and unsaturated ketones, alcohols, and aldehydes on 5 stationary phases in 70–130° range.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

ACKMAN, R. G. AND BURGHER, R. D.: Quantitative gas liquid chromatographic estimation of volatile fatty acids in aqueous media. *Anal. Chem.*, 35 (1963) 647–652 — C_2–C_6 acids at 105°; tailing reducer: formic acid vapour in carrier gas; flame ionization detector; silicone oil, NPGA, Tween.

BISHOP, D. G. AND STILL, J. L.: Fatty acid metabolism in *Serratia marcescens*. III. The constituent fatty acids of the cell. *J. Lipid Res.*, 4 (1963) 81–85 — retention values of fatty acid methyl esters on Apiezon L at 197°; on polyethylene glycol succinate polyester up to 220°.

CASON, J. AND SCHMITZ, F. J.: Epimerization of *s*-dimethylsuccinic acids and derivatives. Cadmium reactions on the ester acid chlorides. *J. Org. Chem.*, 28 (1963) 555–561 — separation of isomeric derivatives on high vacuum silicone grease at 150°.

CRAIG, B. M., TULLOCH, A. P. AND MURTY, N. L.: Quantitative analysis of short chain fatty acids using gas–liquid chromatography. *J. Am. Oil Chemists' Soc.*, 40, No. 2 (1963) 61–63 — butyl esters of C_3–C_9 acids on SE-30 at 180°; above C_9 and dicarboxylic acids at 225°.

GOTTFRIED, E. L. AND RAPPORT, M. M.: The biochemistry of plasmalogens II. Hemolytic activity of some plasmalogen derivatives. *J. Lipid Res.*, 4 (1963) 57–62 — fatty acid methyl esters on diethylene glycol succinate polyester at 175–185°.

KARMEN, A., WALKER, T. AND BOWMAN, R. L.: A sensitive method for quantitative microdetermination of lipids. *J. Lipid Res.*, 4 (1963) 103–106 — lipids dissolved in solvent injected in GC column with uncoated support; after evaporation of solvent, temperature to 660° and flame ionization detection of residual materials.

McDONALD, R., MAY, D., HINDIN, E. AND DUNSTAN, G.: Analysis of volatile organic acids by gas chromatography. *Abstr. 1-R, Div. Water Waste Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — up to C_5 acids; flame ionization.

McGREGOR, R. F., NEWLAND, J. AND CORNATZER, W. E.: Fatty acid composition of milk phospholipids of mice with and without the mammary tumour virus. *Nature*, 198 (1963) 482–483 — C_{14}–C_{16} acids on polyethylene glycol succinate polyester at 195°.

NONAKA, M. AND PIPPEN, E. L.: Gas chromatography of frid chicken volatiles. The effect of storage on composition of the volatile fraction. *Abstr. 17-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

PRIVETT, O. S. AND BLANK, M. L.: A method for structural analysis of triglycerides and lecithins. *J. Am. Oil Chemists' Soc.*, 40, No. 2 (1963) 70–75 — reductive ozonolysis and thin-layer chromatography plus GC; polyethylene glycol succinate polyester at 180°.

ROBINSON, J. D., BRADLEY, R. M. AND BRADY, R. A.: Biosynthesis of fatty acids. III. Utilization of substituted acyl coenzyme A derivatives as intermediates. *J. Biol. Chem.*, 238 (1963) 528–532 — mixed phase of 10% stearic acid + 10% orthophosphoric acid + 80% silicone Dow Corning 550 at 226°; diethylene glycol succinate polyester at 194°.

SHIMO, K. AND WAKAMATSU, S.: Organic syntheses with anion exchange resins. I. The malonic ester synthesis and related reactions. *J. Org. Chem.*, 28 (1963) 504–506 — malonic esters on silicone Dow Corning 550 at 200°.

WILLIAMS, C. M. AND LEONARD, R. H.: Microanalytical determination of dihydroxy aromatic acids by gas chromatography. *Anal. Biochem.*, 5 (1963) 362–366 — as trifluoroacetic acid methyl esters; retention times at 190° on SE-30.

13. STEROIDS

DEPAOLI, J. C., NISHIZAWA, E. AND EIK-NES, K. B.: Hydrolysis of conjugated 17-ketosteroids. *J. Clin. Endocrinol. Metab.*, 23 (1963) 81–89 — androsterone derivatives on G-12 UCON 50 Hb at 200°.

LIN, T. H., RUBINSTEIN, R. AND HOLMES, W. L.: A study of the effect of D- and L-triiodothyrosine on bile acid excretion of rats. *J. Lipid Res.*, 4 (1963) 63–67 — bile acid methyl esters on SE-52/0 at 250°.

15. TERPENE DERIVATIVES

ATTAWAY, J. A., WOLFORD, R. W. AND ALBERDING, G. E.: Identification of alcohols and volatile organic acids from natural orange essence. *Abstr. 18-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

CAMPBELL, J. K., RHOADES, J. W. AND GROSS, A. L.: Acetonitrile as a constituent of cigarette smoke. *Nature*, 198 (1963) 991–992 — GC separation and mass spectrometer identification.

DEADY, L. W., LEARY, G. J., TOPSON, R. D. AND VAUGHAN, J.: N-Arylazetidines. *J. Org. Chem.*, 28 (1963) 511–514 — Apiezon J at 180°.

24. ORGANIC SULPHUR COMPOUNDS

CATES, V. E. AND MELOAN, C. E.: Separation of sulfoxides by gas chromatography. *Anal. Chem.*, 35 (1963) 658–660 — retention values of 13 sulfoxides on Carbowax 1500 or 2M at 200° and 250°.

25. ORGANIC PHOSPHORUS COMPOUNDS

RAUHUT, M. M. AND SEMSEL, A. M.: Reactions of elemental phosphorus with organometallic compounds and alkyl halides. The direct synthesis of tertiary phosphines and cyclotetraphosphines. *J. Org. Chem.*, 28 (1963) 473–477 — differentiation of phosphines on Apiezon L at 244°.

26. METALLO-ORGANIC COMPOUNDS

TIMMS, P. L. AND PHILLIPS, C. S. G.: The study of silicon and germanium hydrides by gas chromatography. *GC Discussion Group Meeting, London, April 5, 1963*; from *Nature*, 198 (1963) 742 — plots of log V_g/number of Si or Ge give the so-called "hydrogen point".

30. INSECTICIDES AND OTHER PESTICIDES

BECKMAN, H. AND BEVENUE, A.: Analysis of grapes and cottonseeds for chlorobenzilate residues. *Abstr. 8-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

BECKMAN, H. AND BEVENUE, A.: Nematocide analysis by gas chromatography with electron capture detector. *Abstr. 8-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — 10 p.p.b. of tetrachlorothiophene and 1,2-dibromo-3-chloropropane in a few mg of crops.

CHALLACOMBE, J. A. AND MCNULTY, J. A.: Applications of the microcoulometric titrating system as a detector in gas chromatography of pesticide residues. *Abstr. 3-A, Div Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

CLARK, S. J.: Quantitative determination of pesticide residues by electron absorption chromatography. *Abstr. 2-A–3-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

GASTON, L. K.: Theoretical aspects of the application of gas chromatography to residue problems. *Abstr. 2-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — detection limits of thermal conductivity; microcoulometric titration: electron affinity = $1:10^3:10^8$.

HARTMANN, H. AND DIMICK, K. P.: Gas chromatography and electron capture for the analysis of pesticides. *Abstr. 2-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles. Calif., March 31–April 5, 1963* — electron capture detector operating properties for p.p.b. range.

LANGLOIS, B. E., STEMP, A. R. AND LISKA, B. J.: Rapid cleanup of dairy products for chlorinated insecticide residue analysis. *Abstr. 7-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — 10–50 p.p.b. lindane, heptachlor, dieldrine, DDT and endrin.

OTT, D. E. AND GUNTHER, F. A.: Forced volatilization cleanup of butterfat for gas chromatographic evaluation of organo-chlorine insecticide residues. *Abstr. 7-A, Div. Agr. Food Chem. 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963.*

SEGAL, H. S. AND SUTHERLAND, M. L.: Comparison of flame ionization and electron capture detectors for the gas chromatographic evaluation of herbicide residues. *Abstr. 4-A, Div. Agr. Food Chem., 144th Meeting Am. Chem Soc., Los Angeles, Calif., March 31–April 5, 1963* — electron capture limit 1 p.p.b. flame ionization 10 p.p.b.

VAN MIDDELEM, C. H. AND WAITES, R. E.: Gas chromatographic and colorimetric measurements of dimethoate residues. *Abstr. 8-A, Div. Agr. Food Chem., 144th Meeting Am. Chem. Soc. Los Angeles, Calif., March 31–April 5, 1963* — microcoulometric detection.

31. PLASTICS AND THEIR INTERMEDIATES

HARWOOD, H. J., BAIKOWITZ, H. AND TROMMER, H. F.: The investigation by vapor phase chromatography. *Abstr. 7-Q, Div. Polymer Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31–April 5, 1963* — styrene–methyl methacrylate polymerization.

KARR, JR., C., COMBERIATI, J. R. AND WARNER, W. C.: Comparison of pitch resins from different sources by combined pyrolysis and gas–liquid chromatography. *Abstr. 5-O, Div. Petroleum Chem., 144th Meeting Am. Chem. Soc., Los Angeles, Calif., March 31-April 5, 1963.*

34. RADIOACTIVE COMPOUNDS

CARTER, JR., E. H. AND SMITH, H. A.: Separation and detection of hydrogen, tritium hydride, and tritium at low-level tritium activities by gas chromatography. *J. Phys. Chem.*, 67 (1963) 535–536.

HILLEN, L. W. AND THACKRAY, M.: Determination of neutron-produced gases in beryllium by gas chromatography. *J. Chromatog.*, 10 (1963) 309–313 — H_2 and He in extracted gases; device described.

35. MISCELLANEOUS OR COMPLEX MIXTURES

BRUNER, F. AND CARTONI, G. P.: Gas chromatographic separation of benzene and deuterobenzenes. *J. Chromatog.*, 10 (1963) 396 — separation of C_6D_6, $C_6H_2D_4$, $C_6H_3D_3$ and C_6H_6 at 10°; (glass capillary 250 m, 0.28 mm I.D.; N_2).

CAVE, G. C. B.: The collection and analysis of odorous gases grom Kraft pulp mills. Part. III. The analysis of collected pollutants by gas chromatography. *Tappi*, 46 (1963) 11–14 — separation of mercaptans, ketones, esters and *n*-alcohols.

CROPPER, F. R. AND KAMINSKY, S.: Determination of toxic organic compounds in admixture in the atmosphere by gas chromatography. *Anal. Chem.*, 35 (1963) 735–743 — trace compounds are absorbed in a small column (E-301; Carbowax 400) and desorbed by heating in a gas chromatograph with flame ionization detectors; retention values of 15 aromatic compounds.

MAHER, T. P.: Gas chromatography applied to the study of the liquid products of the low-temperature fluidized-bed carbonization of coal. *J. Chromatog.*, 10 (1963) 324–337 — analysis of phenolic and neutral fractions C_7–C_{13} on silicone DC 550, Apiezon L up to 220°.

NELSEN, F. M. AND GROENNINGS, S.: Determination of carbon in hydrogen peroxide by combustion-gas chromatography. *Anal. Chem.*, 35 (1963) 660–663 — 900°, O_2 from H_2O_2 oxidizes organic matter to CO_2, which is measured by GC; errors $\pm$ 5% in 100–200 p.p.m.; details of combustion unit.

TERENTIEV, A. P., TURKELTAUB, N. M., BONDAREVSKAYA, E. A. AND DOMOCHKINA, L. A.: (Gas-chromatographic estimation of nitrogen and oxygen in organic compounds). *Dokl. Akad. Nauk SSSR*, 148 (1963) 1316–1319.

THE STANDARDIZATION OF GAS-LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF SIMPLE HYDROCARBON MIXTURES

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INTRODUCTION

The technique of gas-liquid chromatography (GLC), first described by JAMES AND MARTIN[1] in 1952, has been developed at a phenomenal rate but, although so much has been done to advance the knowledge of underlying principles and to devise new techniques and apparatus, little has been done to standardize procedures, particularly with regard to obtaining quantitative results of acceptable precision.

For research purposes, apparatus and techniques can be adapted to fit the special requirements of the research project on hand. Standardization of methods, therefore, is neither necessary nor desirable for this purpose. It is for routine analysis that standardization is required since, for commercial purposes, it is highly desirable that both seller and buyer base their interpretation of analytical results on the same prescribed limits of precision for the analytical tests used.

Up to the present, the techniques of GLC have not been used to any great extent for commercial purposes in the benzole industry, but it is visualized that in the future such methods will be required for testing, both during manufacture and in connection with specifications.

A number of principles and assumptions have been stated in the literature and a number of types of equipment, commercially produced and otherwise, are available. The design of the most suitable equipment and selection of the best operating technique depends ultimately on the validity or otherwise of the underlying assumptions, and it is to test these that the present series of experiments has been designed.

It seems desirable, therefore, to summarize and discuss the more important assumptions so that they can be considered when drawing conclusions from the experiments. The main factors involved are as follows.

(1) *Peak area*

The peak area is a measure of the weight of the constituent responsible for it. The proportion of that constituent in the mixture may be estimated either by referring its peak area to the total area of the chromatogram, or by reference to an added

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* The International Conference of Benzole Producers, which represents all the major producers of aromatic hydrocarbons from coal sources in Europe, has carried out a series of co-operative tests, summarized in this paper, with a view to standardizing techniques for the analysis of aromatic hydrocarbon mixtures.

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standard. The former method is only of use for relatively simple mixtures where no component is present in very large or very small amounts.

Simple proportionality of areas will, in general, not be satisfactory as the weight per unit area differs from component to component. The following are some of the sources of error that may be involved in basing the analysis solely on the ratio of areas.

(a) Calculation of the total area is laborious and difficult with complex mixtures, especially if some of the peaks are small or if some substances are not completely resolved, *i.e.*, if the peaks for these substances overlap.

(b) If some substances are eluted late they may not be recorded on the chromatogram and their areas will not be included in the total area.

(2) *Response factors*

The response factor, *i.e.*, the weight of component per unit area of peak, may vary from compound to compound. The response factors can be determined for each of the substances concerned in an analysis and for the apparatus and conditions used. These response factors enable the composition to be determined from peak areas but, owing to the difficulties of assessing the area representing all the components, it is usual to relate all the areas to the area of an added internal standard. This standard must have a high degree of purity, a retention time such that it will appear on the chromatogram at a point free from other peaks and be used in such quantity that it produces a peak of similar size to those being determined. It should be noted that pre-calibration is necessary for most types of apparatus, and also that the response factor may vary according to the weight of substance introduced into the column. It is usual to operate the GLC equipment under standard conditions of temperature, gas flow and quantity of sample injected. As it is necessary to calibrate the column, however, it is clear that the precision of any estimation must depend upon the variation of the response factors with random variations in the standard operating conditions and upon systematic changes in conditions between the time of making the calibration and making the actual determination.

(3) *Measurement of peak area*

The area of a peak is generally measured by one of the following methods:

(a) Construction of the equivalent triangle, by drawing lines through the points of inflexion on the sides of the peak. The area is proportional to the width of this triangle, measured on the base line, multiplied by the height of the triangle to the apex. This method involves geometric construction, which is subject to personal judgement, particularly with regard to location of the apex of the triangle. Moreover, it has been shown that the relative area of peaks, assessed in this manner, varies according to the size of the peaks. Thus the method is fundamentally unsound if applied to analyses that may involve the measurement of peaks of widely varying magnitude.

(b) Provided the peaks are completely resolved, it is more accurate to multiply the peak height to the peak maximum by the peak width at half peak height. When the peaks overlap, it is necessary to estimate both peak height and peak width, making due allowance for the effect of one peak on the height and width of the other.

(c) The use of a planimeter is capable of giving precise assessment of the areas of peaks, but this method is more tedious and requires greater skill on the part of the operator than measurement of peak heights only. Also, only a high-quality instrument is satisfactory.

(d) Integration of areas. The use of integrating recorders probably gives the most accurate measurement of peak area. If any peaks are not completely resolved, however, the area recorded will be the sum of the areas of the overlapping peaks.

(e) Retention distance. It has been found that over a limited range the width of a peak is approximately proportional to the retention distance. Thus the peak area is approximately proportional to the peak height multiplied by retention time. Although this relationship varies with retention distance, the proportionality factor is included in the calibration. The advantage of this method of calculation is that the retention distance can be measured with considerable precision and thus the criterion, peak height multiplied by retention distance, is more exact than a criterion based on the measurement of peak width.

4. *Peak height*

From what has been said above, it is clear that calibration of the column will generally be needed when precise results are required. Calibration in terms of peak heights, instead of peak areas, would have the advantage of needing one measurement instead of two and would thus lead to greater precision and a saving in time. Difficulties arise when peaks are not completely resolved, because under these conditions the peak height must be estimated.

The following are the usual objections to the use of peak height as a criterion[2]:

(a) Peak area is more fundamental.

(b) Peak height is subjected to greater variation, according to changes in operating conditions, than peak area.

The second objection is of no importance if it can be shown that changes in conditions do not seriously affect the *relative* peak heights. Provided that it can also be shown that analyses based on peak height are as precise as those based on peak area, the greater convenience of making only the single measurement of peak height should outweigh all other considerations.

THE PRESENT TESTS

(1) *General*

In the present series of test, nine laboratories took part, representing the following European countries: Belgium, France, Germany, Great Britain, Italy and the Netherlands. Each laboratory used the equipment and operating conditions available, thus the statistical values for reproducibility, *i.e.* variations between laboratories, will include the effects of different column sizes, different supports, stationary phases, carrier gases, gas speeds and operating temperatures and pressures.

The same materials were used by all the laboratories for calibrating the column and as internal standards. Also, samples of the same unknown mixtures were examined in each laboratory.

Analyses were based on the criteria given in Table I.

TABLE I

METHODS OF ASSESSMENT OF CHROMATOGRAMS

(a) Calibration curves of peak height relative to a standard, against quantity of each substance.
(b) Average relative response factor for peak height.
(c) Calibration curves of peak height multiplied by retention time, relative to a standard, against quantity of each substance.
(d) Average relative response factor for peak height multiplied by retention time.
(e) Calibration curve of peak height multiplied by the peak width at half the peak height, relative to a standard, against quantity of each substance.
(f) Average relative response factor for peak height multiplied by the peak width at half the peak height.

(2) *Apparatus used*

As already stated, no attempt was made to standardize either the type of apparatus used, the liquid phase, or the operating conditions. These ranged according to individual choice. A summary of the equipment and conditions is given in Table II.

(3) *Test and results*

The following pure hydrocarbons for calibration and standards were sent to each laboratory:

Benzene	Cyclohexane	*n*-Octane	(Standard for Test Sample 1)
Toluene	*p*-Xylene	Iso-octane	(Standard for Test Sample 2)
Ethylbenzene	*n*-Nonane		

Calibrations of the apparatus were made as follows:

(a) Benzene, together with 0.3 % w/w, 0.6 % w/w or 1.0 % w/w of toluene, ethylbenzene, cyclohexane and *n*-octane (standard).

(b) Toluene, together with 2.0 % w/w, 4.0 % w/w or 6.0 % w/w of benzene, ethylbenzene and *p*-xylene plus 0.5 % w/w, 1.0 % w/w or 1.5 % w/w *n*-nonane and 0.7 % w/w, 1.5 % w/w or 2.0 % w/w of iso-octane (standard).

Measurements were made of the peak heights, retention distances, and peak widths at half peak height. Calibration graphs were prepared in which the appropriate ratio between the measurements for a constituent and that for the standard, after

TABLE

APPARATUS AND

	Laboratory			
	A	*B*	*C*	*D*
Manufacturers	Perkin-Elmer/ Home	Carlo Erba	Perkin-Elmer	Home
Material of construction	Copper	Stainless steel	Stainless steel	Copper
Solid support	Kieselguhr	Celite C22	Chromosorb	J.M. C22 brick
Liquid phase	Squalane 20 %	PEG 4000 20 %	DEH Sebacate 20 %	DNP 15 %
Detector	Katharometer Hotwire	Katharometer Hotwire	Katharometer Thermistor	Flame
Carrier gas	Helium	Hydrogen	Helium	75 % H_2/25 % N_2
Column temperature (°C)	128	65	150	118
Method of injection	Hamilton syringe	Hamilton syringe	Hamilton syringe	Micro pipette

correction for the quantity of standard, were plotted against concentration in the calibrating mixture free from standard. For peak heights, for example, the ratio:

$$\frac{p_t \times S \times 100}{p_s\,(100\text{-}S)} \text{ was plotted against } \frac{100\,t}{(100\text{-}S)}$$

where p_t and p_s are the peak heights for toluene and standard, t and S are the quantity of toluene and standard in the calibrating mixture. Separate calibration graphs were prepared for each of the six criteria mentioned above under "The Present Tests: (1) General".

Test Samples 1 and 2, the compositions of which are shown in Table III, were then analysed in duplicate in each laboratory and the peak heights and widths at half peak heights and retention distances were reported. The compositions of the two unknown samples were then assessed from these data, according to the six methods (a) to (f). The results are given in Tables III to VIII inclusive.

For each of the methods of assessment of the composition of Samples 1 and 2, a statistical calculation has been made of the Repeatability R_T and the Reproducibility R_{DI}. These may be defined as follows:

Repeatability R_T is the difference between duplicate results, on the same sample, by one operator, using one set of apparatus, that would be equalled or exceeded, in the long run, in only one case in twenty.

Reproducibility R_{DI} is the difference between a single result by one operator at one laboratory and a single result on the same sample by another operator at another laboratory, that would be equalled or exceeded, in the long run, in only one case in twenty.

No significant differences were found in the precision of the determination of the components within Sample 1, but for Sample 2 it was found that the results for *n*-nonane had to be considered separately. Overall precision figures have therefore been calculated for Sample 1 and for Sample 2 excluding *n*-nonane. The precision figures for Sample 1 are applicable to impurities present in quantities less than 1 % and those for Sample 2 for the range 1.5–4 %. These results are given in Table IX.

The following points emerge from an examination of this table:

II

CONDITIONS

Laboratory				
E	*F*	*G*	*H*	*I*
Griffin & George	Carlo Erba	Carlo Erba	Perkin-Elmer/ Home	Carlo Erba
Glass	Stainless steel	Copper	Copper	Stainless steel
Celite 545	Chromosorb P	Celite C22	Chromosorb	A.W. C22
DNP	DNP	Carbowax 1500	D. Iso D.P.	Reoplex 400
20 %	30 %	25 %	20 %	10 %
Katharometer	Katharometer	Katharometer Thermistor	Katharometer	Katharometer Thermistor
Nitrogen	Helium	Hydrogen	Helium	Hydrogen
116	100–120	80	134	110
Agla syringe	Hamilton syringe	Hamilton syringe	Hamilton syringe	Hamilton syringe

TABLE III

RESULTS (% w/w) OBTAINED WITH METHOD (a) FROM PEAK HEIGHTS AND CALIBRATION CURVE

Sample	Theory	Laboratory								
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Sample 1										
Benzene	98.04	97.99	98.22	98.06	98.06	98.06	97.84	98.21	98.06	98.17
		98.02	97.94	98.08	98.04	98.00	97.85	98.15	98.11	98.10
Toluene	0.73	0.76	0.68	0.72	0.71	0.72	0.78	0.63	0.72	0.70
		0.75	0.78	0.72	0.71	0.72	0.78	0.66	0.70	0.73
Ethylbenzene	0.80	0.81	0.73	0.80	0.78	0.80	0.91	0.76	0.80	0.73
		0.80	0.87	0.79	0.78	0.83	0.91	0.77	0.78	0.77
Cyclohexane	0.43	0.44	0.38	0.42	0.45	0.42	0.47	0.40	0.42	0.40
		0.43	0.41	0.41	0.47	0.45	0.46	0.42	0.40	0.40
Sample 2										
Toluene	90.64	90.20	93.13	90.48	90.09	90.79	92.60	91.11	91.08	91.20
		90.92	93.28	90.42	90.15	89.79	92.43	91.12	91.01	91.19
Benzene	1.82	1.90	1.36	1.87	1.96	1.78	1.40	1.70	1.86	1.75
		1.82	1.30	1.88	1.93	1.92	1.42	1.70	1.88	1.75
Ethylbenzene	2.69	2.88	2.10	2.75	2.74	2.70	2.20	6.10*	2.52	2.56
		2.65	2.02	2.77	2.74	2.95	2.25		2.52	2.50
p-Xylene	3.72	3.98	2.66	3.74	4.00	3.65	3.00	6.10*	3.48	3.43
		3.65	2.64	3.77	3.96	4.15	3.10		3.51	3.50
n-Nonane	1.13	1.05	0.75	1.16	1.21	1.08	0.80	1.09	1.06	1.06
		0.95	0.76	1.16	1.22	1.19	0.80	1.08	1.07	1.06

* Ethylbenzene and *p*-xylene not resolved.

TABLE IV

RESULTS (% w/w) OBTAINED WITH METHOD (b) FROM MEAN RELATIVE RESPONSE FACTORS BASED ON PEAK HEIGHTS

Sample	Laboratory								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Benzene	98.00	98.25	98.05	98.04	98.05	97.84	98.16	98.05	98.05
	98.00	97.93	98.08	98.01	97.98	97.85	98.11	98.10	98.03
Toluene	0.75	0.70	0.72	0.71	0.73	0.77	0.62	0.71	0.74
	0.75	0.80	0.72	0.72	0.74	0.77	0.66	0.70	0.74
Ethylbenzene	0.80	0.67	0.81	0.79	0.83	0.93	0.82	0.81	0.81
	0.80	0.86	0.79	0.79	0.88	0.93	0.82	0.79	0.81
Cyclohexane	0.45	0.38	0.42	0.46	0.39	0.46	0.40	0.43	0.40
	0.45	0.41	0.41	0.48	0.40	0.45	0.41	0.41	0.40
Toluene	89.87	93.03	90.50	90.59	90.76	92.33	—	91.00	91.07
	90.62	93.13	90.41	90.66	89.71	92.13	—	90.92	91.08
Benzene	1.89	1.45	1.85	2.01	1.76	1.48	—	1.83	1.76
	1.80	1.35	1.87	1.98	1.94	1.51	—	1.86	1.75
Ethylbenzene	2.85	1.99	2.75	2.74	2.66	2.23	—	2.58	2.58
	2.63	1.99	2.77	2.73	2.92	2.29	—	2.59	2.56
p-Xylene	4.31	2.73	3.75	3.57	3.76	3.01	—	3.53	3.44
	3.97	2.73	3.79	3.53	4.26	3.14	—	3.56	3.47
n-Nonane	1.08	0.80	1.15	1.09	1.06	0.95	—	1.06	1.15
	0.98	0.80	1.16	1.10	1.17	0.93	—	1.07	1.14

TABLE V

RESULTS (% w/w) OBTAINED WITH METHOD (c) FROM PEAK HEIGHTS × RETENTION DISTANCE AND CALIBRATION CURVE

Sample	*Laboratory*								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Benzene	97.99	97.87	98.06	98.13	98.05	97.78	98.20	98.04	98.10
	98.00	97.96	98.10	98.06	98.00	97.78	98.16	98.10	98.14
Toluene	0.76	0.84	0.72	0.70	0.74	0.78	0.64	0.73	0.72
	0.76	0.79	0.72	0.71	0.74	0.79	0.66	0.71	0.71
Ethylbenzene	0.82	0.81	0.80	0.82	0.80	0.98	0.76	0.80	0.78
	0.82	0.85	0.77	0.84	0.84	0.98	0.76	0.78	0.75
Cyclohexane	0.43	0.48	0.42	0.35	0.41	0.46	0.40	0.43	0.40
	0.42	0.40	0.41	0.39	0.42	0.45	0.42	0.41	0.40
Toluene	90.27	93.62	90.47	90.47	90.57	92.36	—	90.98	98.02
	91.00	93.69	90.37	89.94	89.64	91.89	—	90.90	98.21
Benzene	1.86	1.38	1.90	1.95	1.81	1.46	—	1.89	1.74
	1.77	1.28	1.92	2.00	1.96	1.50	—	1.91	1.68
Ethylbenzene	2.80	1.80	2.73	2.68	2.76	2.25	—	2.54	2.64
	2.57	1.82	2.76	2.91	2.99	2.47	—	2.55	2.58
p-Xylene	3.94	2.56	3.74	3.75	3.76	3.01	—	3.51	3.45
	3.62	2.56	3.78	3.93	4.19	3.21	—	3.54	3.41
n-Nonane	1.13	0.64	1.16	1.15	1.10	0.92	—	1.08	1.15
	1.04	0.65	1.17	1.22	1.22	0.93	—	1.10	1.12

TABLE VI

RESULTS (% w/w) OBTAINED WITH METHOD (d) FROM MEAN RELATIVE RESPONSE FACTORS BASED ON PEAK HEIGHTS × RETENTION DISTANCES

Sample	*Laboratory*								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Benzene	98.00	97.84	98.05	98.08	98.04	97.84	98.14	98.09	98.02
	98.01	97.92	98.08	98.02	97.98	97.82	98.10	98.10	98.06
Toluene	0.75	0.87	0.72	0.70	0.74	0.77	0.64	0.71	0.75
	0.75	0.81	0.72	0.71	0.74	0.78	0.66	0.70	0.74
Ethylbenzene	0.80	0.82	0.81	0.77	0.83	0.93	0.82	0.79	0.83
	0.80	0.87	0.79	0.79	0.88	0.94	0.82	0.79	0.80
Cyclohexane	0.45	0.47	0.42	0.45	0.39	0.46	0.40	0.41	0.40
	0.44	0.40	0.41	0.48	0.40	0.46	0.42	0.41	0.40
Toluene	90.31	93.10	90.52	90.66	90.64	92.29	—	90.94	98.98
	91.03	93.18	90.43	90.71	89.63	92.00	—	90.88	91.01
Benzene	1.89	1.43	1.85	2.02	1.77	1.49	—	1.82	1.78
	1.80	1.33	1.87	1.98	1.95	1.52	—	1.85	1.92
Ethylbenzene	2.84	1.96	2.73	2.74	2.70	2.25	—	2.61	2.62
	2.62	1.98	2.76	2.74	2.94	2.32	—	2.62	2.54
p-Xylene	3.88	2.71	3.75	3.49	3.82	3.02	—	3.55	3.46
	3.57	2.71	3.78	3.46	4.30	3.20	—	3.57	3.41
n-Nonane	1.08	0.80	1.15	1.09	1.07	0.95	—	1.08	1.16
	0.98	0.80	1.16	1.11	1.18	0.96	—	1.08	1.12

TABLE VII

RESULTS (% w/w) OBTAINED WITH METHOD (e) FROM PEAK HEIGHTS × PEAK WIDTHS AT HALF PEAK HEIGHT AND CALIBRATION CURVE

Sample	*Laboratory*								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Benzene	98.06	98.21	98.13	98.03	98.10	97.76	98.34	98.10	98.03
	97.96	97.81	98.13	98.09	97.99	97.93	98.35	98.18	98.03
Toluene	0.81	0.74	0.71	0.73	0.71	0.87	0.59	0.81	0.74
	0.78	0.78	0.73	0.69	0.69	0.74	0.66	0.80	0.74
Ethylbenzene	0.86	0.70	0.73	0.81	0.74	0.95	0.67	0.65	0.83
	0.81	1.04	0.72	0.78	0.81	0.92	0.56	0.66	0.83
Cyclohexane	0.37	0.35	0.43	0.43	0.45	0.42	0.40	0.44	0.40
	0.35	0.37	0.42	0.44	0.51	0.41	0.43	0.36	0.40
Toluene	89.94	92.42	90.65	91.06	92.19	92.29	—	91.09	90.96
	90.74	93.04	91.08	91.60	90.50	92.18	—	90.81	90.78
Benzene	2.20	1.76	2.02	1.97	1.65	1.57	—	1.93	1.90
	2.15	1.50	1.83	1.87	1.92	1.57	—	1.95	1.88
Ethylbenzene	3.17	2.16	2.95	2.56	2.23	2.29	—	2.43	2.60
	2.90	2.08	2.59	2.44	2.86	2.36	—	2.61	2.68
p-Xylene	4.13	2.84	3.15	3.29	3.16	2.92	—	3.55	3.36
	3.68	2.58	3.54	3.11	3.79	2.98	—	3.58	3.38
n-Nonane	0.56	0.82	1.23	1.12	0.77	0.93	—	1.00	1.18
	0.53	0.80	0.96	0.98	0.93	0.91	—	1.05	1.28

TABLE VIII

RESULTS (% w/w) OBTAINED WITH METHOD (f) FROM MEAN RELATIVE RESPONSE FACTORS BASED ON PEAK HEIGHTS × PEAK WIDTHS AT HALF PEAK HEIGHT

Sample	*Laboratory*								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Benzene	98.00	98.23	97.98	98.03	97.90	97.76	98.26	98.07	97.99
	98.09	98.12	97.98	98.10	97.81	97.94	98.16	98.14	97.99
Toluene	0.76	0.72	0.76	0.73	0.77	0.81	0.60	0.74	0.76
	0.73	0.70	0.78	0.68	0.72	0.72	0.68	0.72	0.76
Ethylbenzene	0.79	0.69	0.83	0.81	0.88	0.97	0.74	0.77	0.85
	0.75	0.81	0.82	0.78	0.96	0.94	0.73	0.78	0.85
Cyclohexane	0.45	0.36	0.43	0.43	0.45	0.46	0.40	0.42	0.40
	0.43	0.37	0.42	0.44	0.51	0.40	0.43	0.36	0.40
Toluene	90.26	93.23	90.65	91.41	92.26	92.29	—	90.89	91.05
	91.09	93.08	90.47	91.43	90.39	92.39	—	90.61	90.90
Benzene	2.10	1.43	1.84	1.91	1.64	1.56	—	1.88	1.83
	2.04	1.45	1.84	1.87	2.03	1.52	—	1.90	1.81
Ethylbenzene	3.03	1.97	2.69	2.56	2.19	2.36	—	2.57	2.58
	2.68	2.00	2.67	2.44	2.74	2.27	—	2.75	2.67
p-Xylene	4.00	2.54	3.66	3.11	3.10	2.89	—	3.67	3.47
	3.65	2.63	3.68	3.28	3.84	2.88	—	3.70	3.49
n-Nonane	0.61	0.83	1.16	1.01	0.81	0.90	—	0.99	1.07
	0.54	0.84	1.14	0.98	1.00	0.94	—	1.04	1.13

TABLE IX

STATISTICAL ANALYSIS OF RESULTS

Method of assessment	*Sample 1*		*Sample 2*		*Sample 2 (nonane only)*	
	Repeatability R_T	*Reproducibility* R_{DI}	R_T	R_{DI}	R_T	R_{DI}
Peak height						
From curve	0.05	0.10	0.27	1.1	—	—
Mean response (normal)	0.06	0.10	0.32	1.1	0.12	0.52
Mean response (logarithmic transformation)	—	—	13%	39%	—	—
Height × retention distance						
From curve	0.06	0.12	0.33	1.2	0.14	0.74
Mean response	0.05	0.13	0.32	1.0	0.13	0.52
Height × width at half height						
From curve	0.16*	0.23*	0.55	1.0	0.30	1.0
Mean response	0.10	0.17	0.49	1.0	0.18	0.77

* Significantly high

TABLE X

MEAN RESULTS EXPRESSED AS PERCENTAGE OF THE TRUE VALUE FOR SAMPLE I

*Method of assessment**	*Constituent*	*Laboratory* A	B	C	D	E	F	G	H	I	*Mean*
(a)	Toluene	103.4	100.0	98.6	97.3	98.6	106.8	89.7	97.3	97.9	98.6
	Ethylbenzene	100.6	100.0	99.4	97.5	101.9	113.8	95.6	98.6	93.8	100.2
	Cyclohexane	101.2	91.1	96.5	105.8	101.2	108.1	95.3	95.3	93.0	98.6
(b)	Toluene	102.7	102.7	98.6	97.9	100.7	105.5	89.0	96.6	101.4	99.4
	Ethylbenzene	100.0	95.6	100.0	98.8	106.9	116.2	102.5	100.0	101.2	102.4
	Cyclohexane	104.6	91.9	96.5	109.3	91.9	105.8	94.2	97.7	93.0	98.3
(c)	Toluene	104.1	111.6	98.6	96.6	101.4	107.5	89.0	98.6	97.9	100.6
	Ethylbenzene	102.5	103.8	98.1	105.0	102.5	122.5	95.0	98.8	95.6	102.6
	Cyclohexane	98.8	102.3	96.5	86.0	96.5	105.8	95.3	97.7	93.0	97.1
(d)	Toluene	102.7	115.1	98.6	96.6	101.4	106.2	89.0	96.6	102.0	100.9
	Ethylbenzene	100.0	105.6	100.0	97.5	106.9	116.9	102.5	98.8	101.9	103.3
	Cyclohexane	103.8	101.2	96.5	108.1	91.9	107.0	95.3	95.3	93.0	99.1
(e)	Toluene	108.9	104.1	98.6	97.3	97.9	110.3	84.2	110.3	101.4	101.4
	Ethylbenzene	104.4	111.2	90.6	99.4	96.9	116.9	76.9	81.9	103.8	98.0
	Cyclohexane	83.7	83.7	98.8	101.1	111.6	96.5	96.5	93.0	93.0	95.3
(f)	Toluene	102.0	97.3	105.5	96.6	102.1	104.8	87.7	100.0	104.1	100.0
	Ethylbenzene	96.2	93.8	103.1	99.4	115.0	119.4	91.9	96.9	106.2	102.4
	Cyclohexane	102.3	84.9	98.8	101.2	111.6	100.0	96.5	90.7	93.0	97.7
Mean	Toluene	104.0	105.1	99.8	97.0	100.4	106.8	88.1	99.9	100.8	
	Ethylbenzene	100.6	101.7	98.5	99.6	105.0	117.6	94.1	95.9	100.4	
	Cyclohexane	99.1	92.6	97.3	101.9	100.7	103.9	95.5	95.0	93.0	

* See Table I.

TABLE XI

MEAN RESULTS EXPRESSED AS PERCENTAGE OF THE TRUE VALUE FOR SAMPLE 2

*Method of assessment**	*Constituent*	*Laboratory*									*Mean*
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	
(a)	Benzene	102.2	73.1	103.0	106.9	101.6	77.5	93.4	102.7	96.2	95.2
	Ethylbenzene	102.8	76.6	102.6	101.8	105.0	82.7	95.2	93.7	94.0	94.9
	p-Xylene	102.6	71.2	100.9	107.0	104.8	82.0		94.0	93.1	94.4
	n-Nonane	88.5	66.8	102.6	102.6	100.4	70.8	96.0	94.2	93.8	90.6
(b)	Benzene	101.4	76.9	102.2	109.6	101.6	82.1		101.4	96.4	96.4
	Ethylbenzene	101.8	74.0	102.6	101.7	103.7	84.0	**	96.1	95.5	94.9
	p-Xylene	107.0	73.4	101.3	100.8	107.8	82.7		95.3	92.9	95.2
	n-Nonane	91.2	70.8	102.2	96.9	98.7	98.7		93.8	101.3	94.2
(c)	Benzene	99.7	73.1	104.9	108.5	103.6	81.3		104.4	94.0	96.2
	Ethylbenzene	99.8	67.3	102.0	103.9	106.9	87.7	**	94.6	97.0	94.9
	p-Xylene	101.6	68.8	101.1	103.2	106.8	83.6		94.8	92.2	94.0
	n-Nonane	96.0	57.1	103.1	104.9	102.6	81.8		96.5	100.4	92.8
(d)	Benzene	101.4	75.8	102.2	109.9	102.2	82.7		100.8	101.6	97.1
	Ethylbenzene	101.5	73.2	102.0	101.9	104.8	84.9	**	97.2	95.9	95.2
	p-Xylene	100.1	72.8	101.2	93.4	109.1	83.6		95.7	92.3	93.5
	n-Nonane	91.2	70.8	102.2	97.3	99.6	84.5		95.6	100.9	92.8
(e)	Benzene	119.5	89.6	105.8	105.5	98.1	86.3		106.6	103.8	102.0
	Ethylbenzene	112.8	78.8	103.0	92.9	94.6	86.4	**	93.7	98.1	95.0
	p-Xylene	105.0	72.8	89.9	86.0	93.4	79.3		95.8	90.6	83.2
	n-Nonane	48.2	71.7	96.9	92.9	75.2	81.4		90.7	108.8	83.2
(f)	Benzene	113.7	78.3	101.1	103.8	100.8	84.6		103.8	100.0	98.3
	Ethylbenzene	106.1	73.8	99.6	92.9	91.6	86.0	**	98.9	97.6	93.3
	p-Xylene	102.8	69.5	98.6	85.9	93.3	107.2		99.0	93.5	93.7
	n-Nonane	50.9	73.9	101.8	88.0	80.1	81.4		89.8	97.3	82.9
Mean	Benzene	106.3	77.8	103.2	107.4	101.3	81.4		103.3	98.7	
	Ethylbenzene	104.2	74.0	102.0	99.2	101.1	85.3	**	95.7	96.4	
	p-Xylene	104.1	71.4	98.8	96.0	102.5	86.4		95.8	92.4	
	n-Nonane	77.7	68.5	101.5	97.1	92.8	83.1		93.4	100.4	

* See Table I.
** Ethylbenzene and *p*-xylene not resolved

(a) *Repeatability* R_T. For both samples there is no significant difference between the first four methods of assessment but, in general, assessment based on peak height × width at half peak height is less satisfactory. The repeatability for Sample 1 is reasonably satisfactory, but is considerably worse for Sample 2. Contrary to common opinion, therefory, for samples of the type studied, assessment based on peak height only is satisfactory and there is no justification for using more complicated procedures.

(b) *Reproducibility* R_{DI}. As with repeatability, the first four methods of assessment have had no effect on the reproducibility, but values based on peak height × width at half peak height tend to be less precise.

For Sample 1 the reproducibility is about twice the repeatability but, for Sample 2, this ratio has risen to three times.

(c) *Accuracy*. In Tables X and XI the mean values of pairs of results for each constituent and each method of assessment have been calculated as a percentage of the true value. This is a measure of the accuracy of the results; that is of the nearness of the actual result to the true result.

It will be seen that for Sample 1, no analysis shows a serious bias, the analyses having been reasonably accurate and unaffected by the method of assessment (hori-

zontal means). Laboratory F, however, returned high results, those for ethylbenzene being especially high, and Laboratory G returned low results, those for toluene being appreciably low.

For Sample 2, most of the laboratories returned reasonably accurate results. Laboratories B and F returned results, by all methods of assessment, that were appreciably low (vertical means). These anomalous results, which cannot be explained, have worsened the reproducibility and have lowered the overall accuracy. The accuracy is, however, about the same for assessment by all methods, but the results obtained for *n*-nonane tend to be low.

CONCLUSIONS

Analyses based on the measurements of peak height only are as precise as those based on calculation of peak height × retention distance. Analyses based on peak height × width at half peak height, contrary to commonly accepted opinion, tend to be less precise. Thus, for mixtures of the type examined, there is no justification for using the more complicated methods of assessment.

For the first test sample, consisting of benzene containing approximately 2 % w/w of other hydrocarbons, both the precision and accuracy were reasonably satisfactory. For the second sample, however, consisting of toluene containing about 9.5 % w/w of other hydrocarbons, the precision was appreciably worse. The reasons for the poor repeatability are not apparent, but the poor accuracy obtained by two of the participating laboratories has contributed to the poor reproducibility. The accuracy, based on the means of duplicate tests, obtained by the remaining laboratories was good for analyses calculated from peak heights or peak heights × retention distances, but tended to be less satisfactory for analyses based on peak heights × width at half peak height.

Laboratories A, B and G used liquid phases that are generally not considered the best for the types of hydrocarbon mixtures tested, but only one of these, namely Laboratory G, which used Carbowax 1500, produced no separation of ethylbenzene and *p*-xylene. Laboratory B, which used polyethylene glycol, was one of those returning appreciably low results for Sample 2, but there is no evidence that the use of this liquid phase caused the low results. Further work is in progress to elucidate the causes of the poor precision with Sample 2.

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SUMMARY

Nine European laboratories have taken part in a joint exercise in which two samples, one containing about 98 % benzene and the other 90 % toluene, were analysed, using chromatographic equipment available to each laboratory. For each sample, a known quantity of a specified internal standard was used and the composition of the samples

was calculated from calibration data based on (1) peak height, (2) peak height × retention distance, (3) peak height × width of peak at half peak height. The precision of the results is least for method (3), but method (1) is preferred because it is the simplest. The results for the toluene sample were less precise than those for the benzene sample. Further work is in progress.

REFERENCES

[1] A. T. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 50 (1952) 679.
[2] A. I. M. KEULEMANS, *Gas Chromatography*, Reinhold Publishing, New York, 1957, p. 32.

BEITRAG ZUR GASCHROMATOGRAPHISCHEN BESTIMMUNG EINWERTIGER PHENOLE AUS DEM SCHWELTEER

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PROBLEMSTELLUNG

Im Rahmen der Untersuchungen des Schwelteeres unserer einheimischer Xylite aus der Umgebung von Velenje wurde uns die Aufgabe gestellt, die Zusammensetzung der Phenolfraktion aus dem Teer eingehend zu untersuchen. Unser Interesse war vor allem der Identifizierung einzelner Phenole, die in der bis zu 230° siedender Fraktion enthalten sind, gewidmet. Hinsichtlich seiner hohen Trennleistung fanden wir das gaschromatographische Verfahren zu diesem Zweck geradezu ideal. Das Ziel unserer Arbeit war zunächst die für die Trennung eines Gemisches niedrigsiedender, einwertiger Phenole in Betracht kommenden stationären Phasen auf ihre Trennleistung zu prüfen. Aus den Literaturangaben geht hervor, dass Phenole im allgemeinen öfters Gegenstand gaschromatographischer Untersuchungen waren. Ebenso fehlt es nicht an Veröffentlichungen über gaschromatographische Trennungen der Teerphenole, wobei verschiedene stationäre Phasen zur Anwendung gelangten. Eine eingehende Studie über die gaschromatographische Trennung der Phenole verdanken wir FITZGERALD[1,2], der 26 verschiedene stationäre Phasen hierzu erprobt hat. Als besonders wirksame Phasen wurden Apiezon L und Natriumdodecylsulfonat auf Na_2SO_4 erkannt. Apiezon L wurde ausserdem noch von FRANC[3], IRVINE *et al.*[4] sowie JANÁK *et al.*[5] zur Trennung der Phenole vorgeschlagen. Über die Auftrennung der Teerphenole an Dioctylphthalat berichten KARR *et. al*[6,7]. WRABETZ UND SASSENBERG[8] führten Trennungen der Phenol–Kresol–Xylenol-Gemische an Dinonylphthalat durch, dieselbe stationäre Phase wurde auch von FITZGERALD[1] herangezogen. Zu demselben Zweck wurde dagegen von FRANC[3] Didecylphthalat eingesetzt. Eine gute Auftrennung eines Phenolgemisches kann unter anderem auch an Siliconöl[1,9,10] bzw. an Dimethylpolysiloxan[5,11] erreicht werden. Wie von ECKHARDT UND HEINZE[11] gezeigt wurde, erfolgt an einer Säule von 4 m Länge, die Siliconöl als stationäre Phase enthält, eine ausgezeichnete Trennung sämtlicher isomerer Xylenole.

Eine weitere gaschromatographische Methode zur Trennung der Phenole wurde von PAYN[12] ausgearbeitet, wobei Di-*n*-octylsebacat als stationäre Phase dient. An 2,4-Xylenylphosphat gelang BROOKS[13] die Auftrennung von 2,4- und 2,5-Xylenol, *m*- und *p*-Äthylphenol und einiger anderer Phenolpaare, die eine sehr geringe Differenz der Siedepunkte aufweisen. SASSENBERG UND WRABETZ[14] fanden dagegen in *o*-Phthalsäure-di-(3,3,5-trimethylcyclohexyl)-ester eine ideale Phase, auf welcher ein Gemisch sämtlicher isomerer Kresole, Xylenole und *o*-Äthylphenol einwandfrei in Komponenten getrennt wird.

แผนกห้องสมุด กรมวิทยาศาสตร์
กระทรวงอุตสาหกรรม

Wie schon erwähnt, war zunächst die Aufgabe unserer Arbeit, einige von den angegebenen Verfahren in der Praxis auf ihre Trennleistung zu prüfen. Es war uns von vornherein klar, dass keine von den bisher bekannten Phasen eine Auftrennung sämtlicher einwertiger, niedrigsiedender Phenole aus dem Teer herbeizuführen vermag und dass eine eingehende Analyse eines solchen Phenolgemisches nur durch Kombination gaschromatographischer Trennungen an verschiedenen Phasen ermöglicht wird. Da uns zur Durchführung gaschromatographischer Analysen der Gaschromatograph der Firma Pye zur Verfügung steht und bei diesem Apparat die Länge der Standardsäulen nur etwa 120 cm beträgt, waren wir gezwungen, nur solche stationäre Phasen zu verwenden, an denen möglichst grosse Differenzen der Wanderungsgeschwindigkeiten zwischen je zwei benachbarten Komponenten erreicht werden. Obwohl wir aus zahlreichen Veröffentlichungen über gaschromatographische Phenoltrennungen schon die Wanderungsgeschwindigkeiten für eine grosse Anzahl der Phenolderivate direkt entnehmen konnten, fanden wir die meisten von diesen Angaben insofern als mangelhaft, da die Trennleistung der einzelnen Verfahren nur an einer beschränkten Anzahl der Phenole gezeigt wird. Hingegen fehlen oft die Angaben über andere Komponenten, die in einem Teerphenolgemisch ebenfalls auftreten, jedoch an der betreffenden Phase eventuell ungetrennt mit einer anderen Komponente wandern. Ausserdem kommt noch hinzu, dass wir schon bei unseren ersten Versuchen zur Trennung eines Gemisches der Teerphenole aus unseren einheimischen Xyliten eine neue Komponente entdeckten, die unseres Wissens bisher nicht unter den Teerphenolen erwähnt bzw. gaschromatographisch identifiziert wurde. Durch Kombination der Trennungen an verschiedenen stationären Phasen als auch an Hand papierchromatographischer Untersuchungen stellten wir fest, dass es sich um Guajakol handelt. Somit mussten wir auch diese Komponente, über welche uns die Retentionszeiten auch für die gebräuchlichsten Phasen fehlten, in unsere Versuchsreihe einbeziehen. Die Trennleistungen einzelner gaschromatographischer Verfahren haben wir somit jeweils mit dem Gemisch folgender Phenole überprüft: Phenol, Guajakol, *o*-, *m*- und *p*-Kresol, 2,6-, 2,5-, 2,4-, 2,3-, 3,5- und 3,4-Xylenol, *o*- und *p*-Äthylphenol.

EXPERIMENTELLER TEIL

Apparatives

Sämtliche gaschromatographische Trennungen wurden mit dem Gaschromatographen der Firma Pye (Cambridge, England) durchgeführt. Dieser Gaschromatograph arbeitet mit einem Ionisationsdetektor nach LOVELOCK, als Trägergas wird Argon verwendet.

Säulen

Die Trennungen der Phenole wurden an Standardsäulen von etwa 120 cm effektiver Länge und mit einem inneren Durchmesser von etwa 6 mm durchgeführt.

Trägersubstanz

Zur Bereitung der stationären Phasen diente säuregewaschenes Embacel (May & Baker); Korngrösse 0.15 bis 0.25 mm (60/100 mesh).

Die Zusammensetzung verschiedener stationärer Phasen und die entsprechenden Arbeitsbedingungen sind in der Tabelle I zusammengestellt.

TABELLE I

STATIONÄRE PHASEN UND ARBEITSBEDINGUNGEN

Organische Phase	*Apiezon L*	*Di-nonyl-phthalat*	*2,4-Xylenyl-phosphat*	*Polyäthy-lenglykol-adipat*	*Trimethyl-cyclohexyl-phthalat*
Provenienz	*Wilkens & Co.*	*May & Baker*	*Pye*	*Pye*	*W. Sassenberg und K. Wrabetz*
Konzentration der org. Phase an Embacel (Gew. %)	20*	20**	5***	20	20
Säulentemperatur (°C)	90	130	110	130	130
Trägergas Einlassdıuck (atm)	0.1	0.25	0.2	0.5	0.2
Empfindlichkeitsstufe	3	3	3	3	3
Detektorelektrodenspannung (V)	1500	1500	1500	1500	1500
Papiervorschub (cm/h)	40	40	40	40	40

* Neben 20 % Apiezon L wurde Embacel vorher noch mit 3 % H_3PO_4 imprägniert.
** Die stationäre Phase enthielt zusätzlich noch 3 % H_3PO_4.
*** Neben 2,4-Xylenylphosphat enthielt die Phase noch 1 % H_3PO_4.

Standardsubstanzen

Zur Bestimmung der Wanderungsgeschwindigkeiten einzelner Phenole standen uns Reinsubstanzen folgender Provenienz zur Verfügung (Tabelle II).

Bei gaschromatographischen Trennungen wurden die Phenole jeweils in Form ihrer Lösungen in die Säule eingebracht. Als Lösungsmittel hat sich hierbei Tetrachlorkohlenstoff als sehr günstig erwiesen, da der Ionisationsdetektor gegenüber CCl_4 nur wenig empfindlich ist und der Schreiberausschlag auch bei höherer Lösungsmittelkonzentration innerhalb des Skalenbereiches bleibt.

Unter den in Tabelle I angegebenen Arbeitsbedingungen haben wir zunächst an verschiedenen stationären Phasen die Retentionszeiten für einzelne Reinphenole ermittelt. Die auf diese Weise erhaltenen Ergebnisse sind in der Tabelle III zusammengestellt. Die dort angeführten Retentionswerte gelten für den Fall, dass die Phenole

TABELLE II

PROVENIENZ DER VERWENDETEN REINSUBSTANZEN

Phenolderivat	*Hersteller*
Phenol, chem. rein	Riedel de Haën
Guajakol, reinst	The Coleman & Bell Co.
o-Kresol	The Coleman & Bell Co.
m-Kresol	The Coleman & Bell Co.
p-Kresol	The Coleman & Bell Co.
2,6-Xylenol	Dr. Theodor Schuchardt G.m.b.H.
2,5-Xylenol	Dr. Theodor Schuchardt G.m.b.H.
2,4-Xylenol	Eastman-Kodak
2,3-Xylenol	Durch Dr. SASSENBERG und Dr. WRABETZ zur Verfügung gestellt
3,5-Xylenol	Dr. Theodor Schuchardt G.m.b.H.
3,4-Xylenol	Dr. Theodor Schuchardt G.m.b.H.
o-Äthylphenol	Durch Dr. SASSENBERG und Dr. WRABETZ zur Verfügung gestellt.
p-Äthylphenol	Imperial Chemical Industries Ltd.

vereinzelt, also nicht im Gemisch mit anderen Komponenten, auf den Start aufgetragen werden. Wir stellten nähmlich fest, dass die Wanderungsgeschwindigkeiten der Komponenten — besonders zweier benachbarter Phenole, die nahe beisammen liegen — im Gemisch mit einer grösseren Anzahl anderer Komponenten etwas beeinflusst werden, wodurch unter Umständen eine Trennung auch ausbleiben kann. Aus diesem Grunde konnten wir die Trennleistung einer stationären Phase nicht ohne weiteres an Hand der ermittelten Retentionszeiten für Standardsubstanzen beurteilen, sondern mussten dieselbe praktisch mit dem Gemisch obengenannter 13 Komponenten erproben.

TABELLE III

RELATIVE RETENTIONEN VON PHENOLEN

Substanz	*Bezeichnung*	*Stationäre Phase*				
		Apiezon L	*Dinonylphthalat*	*2,4-Xylenylphthalat*	*Polyäthylenglykoladipat*	*Trimethylcyclohexylphthalat*
Phenol	A	1.00	1.00	1.00	1.00	1.00
Guajakol	B	3.26	0.99	0.52	0.76	0.95
o-Kresol	C	1.99	1.37	1.18	1.08	1.33
m-Kresol	D	2.17	1.79	1.67	1.50	1.82
p-Kresol	E	2.16	1.77	1.57	1.50	1.68
2,6-Xylenol	F	3.81	1.37	0.99	0.92	1.43
2,5-Xylenol	G	4.31	2.44	1.87	1.58	2.44
2,4-Xylenol	H	4.31	2.43	1.88	1.57	2.31
2,3-Xylenol	I	5.18	2.93	2.43	2.05	2.88
3,5-Xylenol	J	4.83	3.26	2.81	2.30	3.34
3,4-Xylenol	K	5.72	3.67	3.14	2.78	3.68
o-Äthylphenol	L	3.50	2.19	1.85	1.42	2.18
p-Äthylphenol	M	4.16	2.94	2.61	2.13	2.98

RESULTATE

1. Stationäre Phase auf Basis von Apiezon L

Apiezon L hat sich zur Trennung der Phenole als sehr günstig erwiesen, da an dieser stationären Phase die Phenole relativ niedrige Rückhaltezeiten aufweisen. Das ist insofern vorteilhaft, da man deshalb bei Temperaturen, die bis 120° unter dem Siedepunkt der Phenole liegen, arbeiten kann. Leider neigen, wie das schon durch andere Autoren festgestellt wurde, die Phenole dazu, an Apiezon asymmetrische Elutionsbanden zu bilden. Diese Erscheinung kann durch Behandlung der Trägersubstanz mit einer nicht flüchtigen Säure (wie etwa Phosphorsäure) weitgehend beseitigt werden. Mit einem Gemisch frisch destillierter Teerphenole erhält man an einer solchen stationären Phase eine relativ gute Auftrennung der Komponenten, wobei die gebildeten Elutionsmaxima weitgehend symmetrisch sind. Beim Auftragen eines Gemisches von Teerphenolen, in welchem auch Oxydationsprodukte oder teils verharzte Komponenten enthalten sind, verliert die Phase rasch an ihrer Wirksamkeit, was an dem Auftreten stark asymmetrischer und unscharfer Elutionsbanden in Chromatogrammen erkannt wird. Das Chromatogramm eines Phenolgemisches an Apiezon L zeigt die Fig. 1.

Aus der Abbildung geht hervor, dass das Gemisch von 13 Phenolen in nur 8

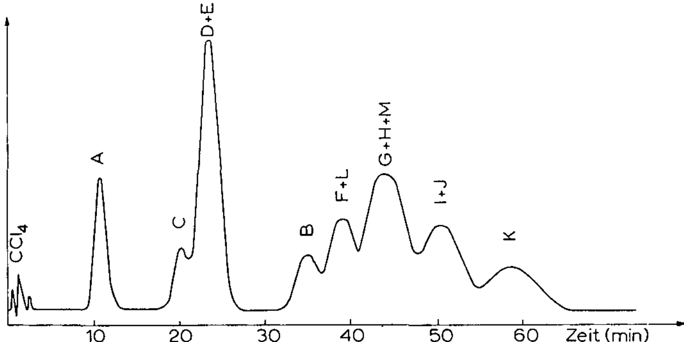

Fig. 1. Chromatogramm eines Gemisches von 13 Phenolen an Apiezon L. Arbeitsbedingungen siehe Tabelle I. Bezeichnung der Komponenten siehe Tabelle III.

Fraktionen getrennt wurde. Ohne Auftrennung wandern *m*-Kresol und *p*-Kresol, 2,6-Xylenol und *o*-Äthylphenol, 2,4-Xylenol, 2,5-Xylenol und *p*-Äthylphenol sowie 3,5-Xylenol und 2,3-Xylenol. Überraschend ist hierbei, dass trotz der relativ grossen Differenz der Retentionen für reines *o*-Äthylphenol und 2,6-Xylenol diese beiden Komponenten im Gemisch ungetrennt wandern.

2. Stationäre Phase auf Basis von Dinonylphthalat

Zum Unterschied zu Apiezon L sind die Rückhaltezeiten einzelner Phenole an Dinonylphthalat verhältnismässig lang. An stationären Phasen, die mit einer grösseren Konzentration von Dinonylphthalat bereitet waren (25-35 %), bilden die Phenole durchaus symmetrische Elutionsmaxima. Nachteilig hierbei ist, dass eine solche Analyse viel Zeit in Anspruch nimmt. Bei unseren Versuchen wählten wir deshalb eine niedrigere Konzentration der organischen Phase an Embacel (20 %) und setzten ausserdem noch 3 % Phosphorsäure hinzu. Wie aus Fig. 2 hervorgeht, wurden auf diese Weise völlig symmetrische Elutionsbanden gebildet.

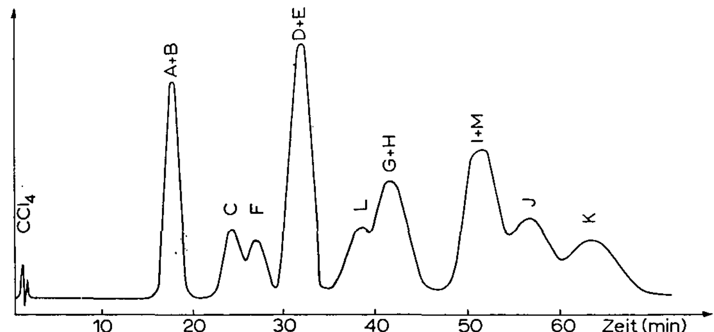

Fig. 2. Chromatogramm eines Gemisches von 13 Phenolen an Dinonylphthalat. Arbeitsbedingungen siehe Tabelle I. Bezeichnung der Komponenten siehe Tabelle III.

Das Gemisch von 13 Teerphenolen wurde auf dieser stationären Phase in 9 Fraktionen getrennt. In einem gemeinsamen Berg wanderten jeweils folgende Phenolpaare: Phenol und Guajakol, *m*-Kresol und *p*-Kresol, 2,4-Xylenol und 2,5-Xylenol sowie 2,3-Xylenol und *p*-Äthylphenol.

3. Stationäre Phase auf Basis von 2,4-Xylenylphosphat

An 2,4-Xylenylphosphat gelang BROOKS[13] die Auftrennung einiger Phenolpaare, die an den gebräuchlichsten Phasen ohne Trennung wandern. Dieses gilt vor allem für *m*-Kresol und *p*-Kresol, 2,4-Xylenol und 2,5-Xylenol sowie *m*-Äthylphenol und *p*-Äthylphenol. An Hand dieser Angaben schien uns eine gute Trennung des Gemisches der Teerphenole an dieser Phase möglich, und setzten wir deshalb dieselbe bei unserer praktischen Arbeit ein. Hierbei stellten wir fest, dass an 2,4-Xylenylphosphat eine teilweise Trennung von *m*-Kresol und *p*-Kresol eintrat, dass *p*-Äthylphenol einwandfrei von 2,3-Xylenol getrennt wird und dass eine Auftrennung von 2,4-Xylenol und 2,5-Xylenol hierbei nicht stattfindet (Fig. 3).

Wir versuchten weiterhin durch Änderung der Konzentration von 2,4-Xylenylphosphat an Embacel, der Arbeitstemperatur und der Durchflussgeschwindigkeit des Trägergases eine Auftrennung des 2,4-Xylenols von 2,5-Xylenol herbeizuführen.

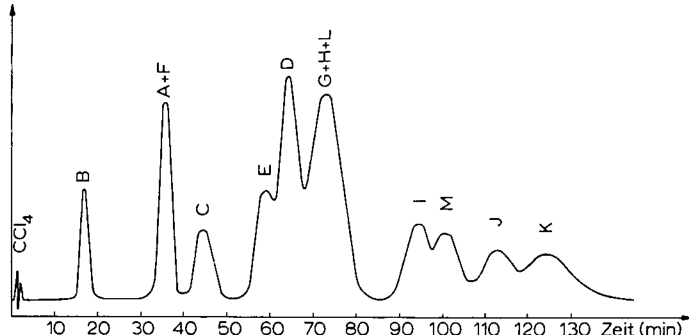

Fig. 3. Chromatogramm eines Gemisches von 13 Phenolen an 2,4-Xylenylphosphat. Arbeitsbedingungen siehe Tabelle I. Bezeichnung der Komponenten siehe Tabelle III.

Leider blieben alle diese Versuche erfolglos. Trotzdem finden wir 2,4-Xylenylphosphat als eine sehr günstige stationäre Phase zur Trennung der Teerphenole, da sie einerseits eine Abtrennung des Guajakols von den übrigen Phenolen, anderseits aber eine gute Trennung des *p*-Äthylphenols von 2,3-Xylenol ermöglicht, was an anderen Phasen nicht erreicht wird. Die Abtrennung des *p*-Kresols vom *m*-Kresol ist an 2,4-Xylenylphosphat nicht ausreichend um eine sichere quantitative Auswertung dieser Komponenten des Phenolgemisches zu ermöglichen. Ebenso bleiben Phenol und 2,6-Xylenol an dieser Phase ungetrennt.

4. Stationäre Phase auf Basis von Polyäthylenglykoladipat

Obwohl Polyäthylenglykoladipat bisher kaum zur Trennung der Phenole Verwendung fand, haben wir diese stationäre Phase bei unserer Arbeit mit herangezogen. Die Retentionswerte der Phenole sind an Polyäthylenglykoladipat relativ hoch, deswegen wählten wir eine grössere Durchflussgeschwindigkeit des Trägergases um nicht die Säulentemperatur unnötigerweise zu steigern. Da an der stationären Phase, die 20 % Polyäthylenglykoladipat an Embacel enthielt, die Elutionsbanden einzelner Phenole praktisch völlig symmetrisch sind, bedarf diese Phase keines Säurezusatzes. Wie aus der Fig. 4 ersichtlich ist, wird das Gemisch von 13 Teerphenolen in 8 Fraktionen getrennt.

Polyäthylenglykoladipat ist als stationäre Phase für die Trennung der Phenole insofern günstig, als sie das Gemisch von Phenol, Guajakol, 2,6-Xylenol und *o*-Kresol vollkommen aufzutrennen vermag. Die Wanderungsgeschwindigkeit des 2,6-Xylenols wird an Polyäthylenglykoladipat sehr vergrössert, so dass 2,6-Xylenol

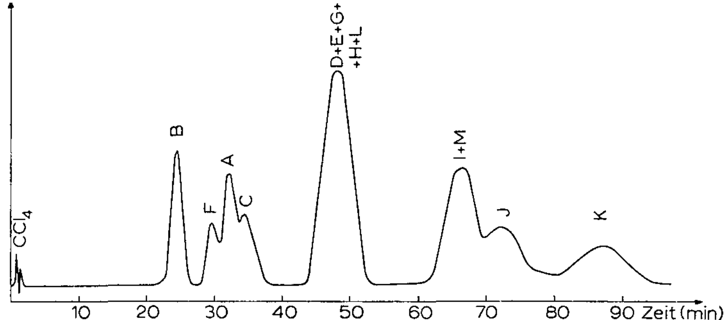

Fig. 4. Chromatogramm eines Gemisches von 13 Phenolen an Polyäthylenglykoladipat. Arbeitsbedingungen siehe Tabelle I. Bezeichnung der Komponenten siehe Tabelle III.

sogar vor dem Phenol wandert. Die Trennleistung dieser Phase gegenüber den anderen Komponenten des Gemisches ist relativ gering, so dass *m*-Kresol, *p*-Kresol, 2,4-Xylenol, 2,5-Xylenol und *o*-Äthylphenol gemeinsam in einem Elutionsmaximum auftreten. Ebenso wird 2,3-Xylenol nicht von *p*-Äthylphenol getrennt.

5. *Stationäre Phase auf Basis von o-Phthalsäure-di-(3,3,5-trimethylcyclohexyl)-ester*

o-Phthalsäure-di-(3,3,5-trimethylcyclohexyl)-ester wurde von SASSENBERG UND WRABETZ[14] als die bisher wirksamste Phase zur Trennung der Phenolderivate vorgeschlagen. An einer Säule von 3 m Länge konnten diese Autoren eine einwandfreie Trennung eines Gemisches von 11 Phenolen erreichen. Obwohl in ihrer Arbeit die Angaben über die Retentionen von 12 Phenolen enthalten sind, haben wir die Wanderungsgeschwindigkeiten dieser Komponenten unter unseren Arbeitsbedingungen erneut ermittelt und dabei auch das Verhalten von Guajakol geprüft. Die in der Tabelle III angegebenen Retentionen stimmen mit den von SASSENBERG UND WRABETZ ermittelten Wanderungsgeschwindigkeiten gut überein, eine kleine Abweichung tritt nur bei *p*-Äthylphenol auf. Phenol und Guajakol zeigen eine nur sehr geringe Differenz der Rückhaltezeiten, so dass mit einer vollkommenen Auftrennung dieser beiden Komponenten im Gemisch nicht gerechnet werden konnte. Die Auftrennung des Gemisches von 13 Phenolen ist aus dem Chromatogramm ersichtlich (Fig. 5).

Auf Trimethylcyclohexylphthalat wurde das Gemisch von 13 Teerphenolen in 11 Fraktionen aufgetrennt. An einer Säule von 120 cm Länge trennte sich Guajakol nicht vollkommen von Phenol, ebenfalls blieb das Gemisch von 2,3-Xylenol und *p*-Äthylphenol ungetrennt. Alle übrigen Phenole sind voneinander deutlich getrennt, dasselbe gilt vor allem für die Paare *m*-Kresol und *p*-Kresol sowie 2,4-Xylenol und 2,5-Xylenol. Somit wurde die hohe Trennleistung dieser stationären Phase noch einmal deutlich bewiesen.

An Hand der durchgeführten Versuche stellten wir also fest, dass an keiner der bisher bekannten stationären Phasen eine vollkommene Auftrennung eines komplexen Gemisches der niedrigsiedenden Teerphenole erreicht wird. Eine komplette

Analyse des Phenolgemisches wird durch Trennungen an mindestens zwei verschiedenen Phasen erreicht. Als solche Phasen schlagen wir *o*-Phthalsäure-di-(3,3,5-trimethylcyclohexyl)-ester und 2,4-Xylenylphosphat vor. An 2,4-Xylenylphosphat wird eine Trennung von 2,3-Xylenol und *p*-Äthylphenol und eine vollkommene Ab-

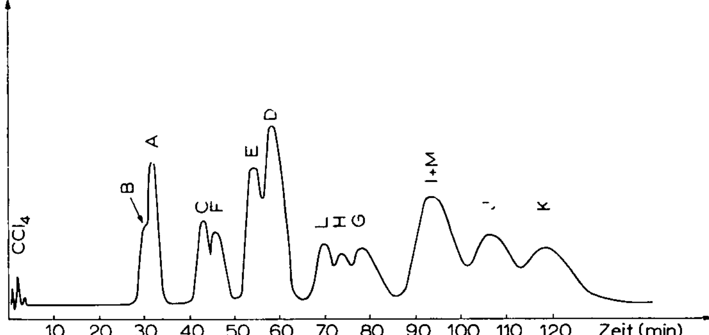

Fig. 5. Chromatogramm eines Gemisches von 13 Phenolen auf Trimethylcyclohexylphthalat. Arbeitsbedingungen siehe Tabelle I. Bezeichnung der Komponenten siehe Tabelle III.

trennung des Guajakols von den übrigen Komponenten des Gemisches herbeigeführt, was an Trimethylcyclohexylphthalat nicht der Fall ist. Die Auftrennung sämtlicher anderen Komponenten des Gemisches erfolgt einwandfrei an Trimethylcyclohexylphthalat.

DANK

Für die freundliche Überlassung der Muster von Trimethylcyclohexylphthalat, 2,3-Xylenol und *o*-Äthylphenol möchten wir Herren Dr. W. SASSENBERG und Dr. K. WRABETZ auch an dieser Stelle unseren herzlichsten Dank zum Ausdruck bringen.

ZUSAMMENFASSUNG

An einem Gemisch von 13 niedrigsiedenden Teerphenolen wurde die Trennleistung verschiedener stationären Phasen überprüft. Unter den Phenolen aus dem Schwelteer einheimischer Xylite wurde auch Guajakol festgestellt. Eine vollkommene Analyse des Gemisches von 13 Komponenten gelingt durch Trennung an mindestens zwei verschiedenen Phasen, wofür das 2,4-Xylenylphosphat und *o*-Phthalsäure-di-(3,3,5-trimethylcyclohexyl)-ester vorgeschlagen werden.

SUMMARY

The separating power of various stationary phases was investigated, using a mixture of 13 low-boiling tar phenols. In the phenols from low-temperature carbonization tar from indigenous xylite, the presence of guaiacol was also established. A complete analysis of a mixture of 13 components is possible by separation on at least two different phases, for which 2,4-xylenyl phosphate and *o*-phthalic acid di-(3,3,5-trimethylcyclohexyl) ester are recommended.

LITERATUR

1 J. S. Fitzgerald, *Australian J. Appl. Sci.*, 10 (1959) 169.
2 J. S. Fitzgerald, *Australian J. Appl. Sci.*, 10 (1959) 306.
3 J. Franc, *Collection Czech. Chem. Commun.*, 25 (1960) 1573.
4 L. Irvine und T. J. Mitchell, *J. Appl. Chem. (London)*, 8 (1958) 425.
5 J. Janák, R. Komers und J. Šíma, *Collection Czech. Chem. Commun.*, 24 (1959) 1492.
6 C. Karr, P. M. Brown, P. A. Estep und G. L. Humphrey, *Fuel*, 37 (1958) 227.
7 C. Karr, P. M. Brown, P. A. Estep und G. Humphrey, *Anal. Chem.*, 30 (1958) 1413.
8 K. Wrabetz und W. Sassenberg, *Z. Anal. Chem.*, 179 (1961) 333.
9 E. C. Jennings, T. D. Curran und D. G. Edwards, *Anal. Chem.*, 30 (1958) 1946.
10 A. Kreyenbuhl und H. Weiss, *Bull. Soc. Chim. France*, 11/12 (1959) 1880.
11 F. Eckhardt und H.-O. Heinze, *Brennstoff-Chem.*, 42 (1961) 136.
12 D. S. Payn, *Chem. Ind.*, *(London)*, 34 (1960) 1090.
13 V. T. Brooks, *Chem. Ind. (London)*, 42 (1959) 1317.
14 W. Sassenberg und K. Wrabetz, *Z. Anal. Chem.*, 184 (1961) 423.

THE EFFECT OF TEMPERATURE AND CARRIER GAS ON THE LOSS RATE OF TRITIUM FROM RADIOACTIVE FOILS*

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INTRODUCTION

In this and other laboratories commonly employing ionization detection devices the source of radiation selected is often the relatively stable hydrogen isotope, tritium, a pure beta emitter. The tritium is usually available as the titanium tritide, with either copper or stainless steel backing. Detailed descriptions of the foils proper have been given by VANDERSCHMIDT *et al.*[1] and WILSON[2]. The tritium betas with a mean energy of 5 keV are not sufficiently energetic to penetrate even a thin layer of restraining material. Bremsstrahlung from these weak betas should be negligible. For these reasons, a source contained within a detection cell should not constitute a hazard due to surface activity at the detector body.

The hazards involved with initial loading and subsequent modification of any ionization device should be of little concern to the average operator since by law this procedure is to be carried out only by qualified, licensed personnel. It has been shown[2] that the action of sample components upon the source will be minor in nature owing to their presence in such minute amounts. The most noticeable action would be from components having a corrosive effect upon the metal backing of the foil. A more detailed treatment of possible hazards arising from use of radioactive foils has been given by TAYLOR[3].

The National Aeronautics and Space Administration contemplate the use of gas chromatography employing ionization detectors in some of their interplanetary probes. Quite often it is necessary to operate these devices under varying conditions. For this reason, it would be desirable to know the effect of temperature and carrier gas upon the stability of these foils since tritium is a very attractive source in the laboratory development program of most of these devices. If losses should occur anomalous results would most likely be encountered without frequent calibration. Further, extreme losses might lead to hazardous conditions unless proper ventilation was provided, due to enrichment of effluent vapor from the system in tritium gas. This paper reports on such a study.

EXPERIMENTAL

The radioactive foil used in these studies was obtained from U.S. Radium Corporation. The metal backing was of copper. The data were obtained from the experimental

* This work was supported under a grant provided by the National Aeronautics and Space Administration.

arrangement shown in Fig. 1. A known flow of clean, dry carrier gas was passed into a glass chamber containing the radioactive foil. The glass chamber was stationed in a furnace capable of elevating the temperature to 300°. The effluent gas was led through a short section of narrow diameter tubing into a large volume ionization chamber and from here to a fume hood.

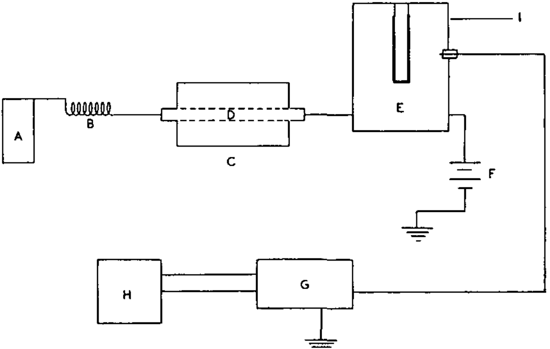

Fig. 1. Experimental arrangement: (A) carrier gas supply; (B) 5 A molecular sieve drying column and capillary restrictor; (C) furnace; (D) glass chamber containing the radioactive foil; (E) ionization chamber; (F) polarizing potential; (G) electrometer; (H) recorder; (I) effluent gas to fume hood.

Fig. 2 illustrates the actual ion chamber used in the experimental operations. The collection electrode is a 3 cm diameter brass tube isolated from the body of the chamber by a teflon supporting rod. The chamber body is a cylindrical brass tube 13.5 cm in diameter. Gas enters the chamber through a gas-tight connection in the lower section and exits through a similar connection in the upper section. The total volume was measured and found to be approximately 2 l. The necessary electrical connections are made through the body of the chamber using gas-tight teflon insulators to minimize electrical leakage. A polarizing potential is developed between the detector body and the collection electrode. The electrical signal generated was fed into a high gain electrometer and subsequently recorded graphically by means of a potentiometric recorder. Details of the considerations to be made in measurements of this type as well as construction details of ionization chambers in general have been given by OVERMAN AND CLARK[4] and Nuclear of Chicago[5].

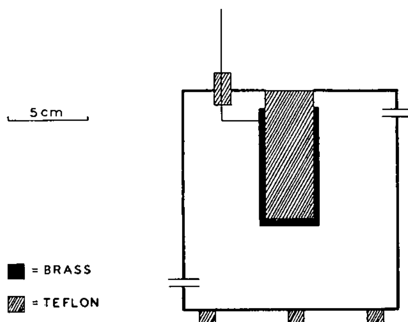

Fig. 2. Ionization chamber. Shaded sections of brass; lined sections of teflon.

A voltage *versus* current relationship was determined to establish the proper operating conditions for the system. Fig. 3 demonstrates that the chamber is operating as a simple ionization chamber at 300 V and that all the ions formed are being collected before there is a chance for recombination to occur. A measured volumetric flow of carrier gas, 100 ml/min, was allowed to pass through the system for several hours until equilibrium was reached. The signal generated indicated a normal back-

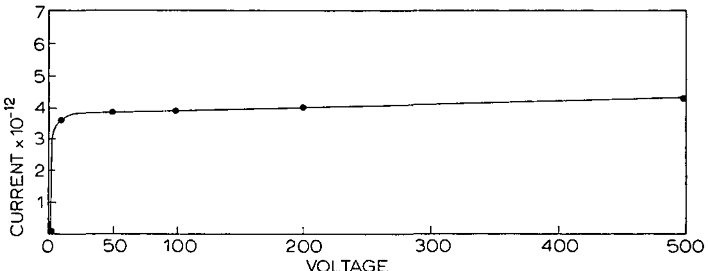

Fig. 3. Voltage *versus* current for the ionization chamber.

ground. The furnace temperature was adjusted to the desired level and several hours were required to reach a steady state equilibrium whereby the amount of tritium gas entering the chamber was equivalent to the amount of tritium gas being expelled. The signal thus obtained indicated the concentration of radioactive species within the chamber.

Tritium betas have a maximum range of about 1 cm in air at S.T.P. The dimensions of the ionization chamber were so arranged that the distance between electrodes far exceeds this value. This would imply that the total energy of the tritium betas should be dissipated in the gas phase. The number of ion pairs formed per beta particle should, to a very close approximation, be equal to the average energy of the tritium beta divided by the specific ionization energy of the gas under consideration. Values for specific ionization energies have been presented by SHARPE[6]. The average value is around 30 eV. Since 1 microcurie (μC) is equal to $3.700 \cdot 10^4$ disintegrations per second, the following relationship may be written:

$$C = \frac{IS(6.281 \cdot 10^{18})}{(5 \cdot 10^3)\,(3.700 \cdot 10^4)}$$

where:

C = concentration of radioactive species (μC),
I = observed current (A),
S = specific ionization energy of the carrier gas (eV).

The rate of loss from the tritium foil is now described by:

$$R = \frac{CF}{V}$$

where:

R = rate of tritium loss (μC/sec),
C = concentration of radioactive species (μC),
F = volumetric flow of carrier gas (cm^3/sec),
V = volume of ionization chamber (cm^3).

A single radioactive foil was used in all the experiments herein reported. Its activity was measured before and after each investigation in terms of the current it provided in a small volume ionization chamber under a standard set of conditions. Allowances were made in the calculations for any change in observed activity.

RESULTS AND DISCUSSION

The gases studied were H_2, He, N_2, air, Ar + 5% CH_4 and Ar saturated with water vapor. The temperature ranged from ambient to 290°. Fig. 4 shows the ion current observed *versus* temperature for the above gases. As might be expected, the current rises very rapidly above 200° indicating an appreciable loss of tritium from the radio-

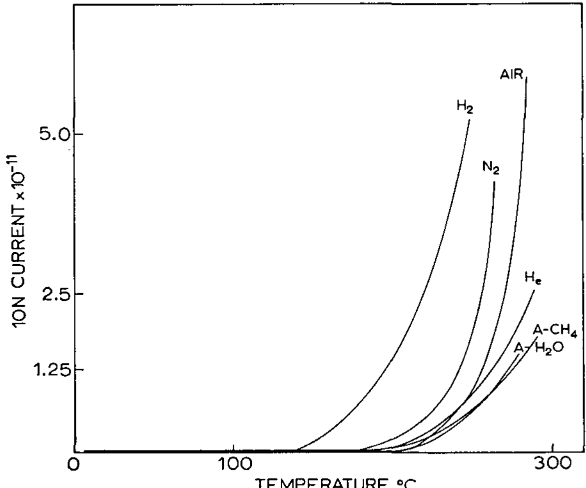

Fig. 4. Ion current *versus* temperature for various carrier gases, before correction for changes in source strength.

active foil. The exception noted was with hydrogen. In this case, noticeable losses occur above 140°. This observation could be accounted for if an exchange occurred between the hydrogen gas and the tritium contained on the foil as titanium tritide. Such an observation would appear to be reasonable since similar observations could not be made with other gases.

Table I lists the calculated loss rate of tritium in various carrier gases with respect to temperature. The loss rate R is given in units of both μC/sec and the more practical unit of mC/day. R is seen to range in value from 10^{-2} to 6 mC/day depending upon the gas used and operating temperature. These values are in close agreement with those given by WILSON[2]. The concentration C, of tritium gas in air, assuming a 1000 cu.ft. room with a turnover rate of 1 volume per hour is presented. The assumed enclosure and ventilation conditions are worse than those normally encountered. Nevertheless, it should be noted that the maximum permissible concentration in air for tritium gas is not exceeded.

Table II gives the calculated half life of an average tritium foil in various gases at the indicated temperatures. This half life is based solely on the loss rate and does not

TABLE I

LOSS RATE OF TRITIUM FOR VARIOUS GASES AT DIFFERENT TEMPERATURES BASED ON A ON A 50 mC SOURCE

Gas	*Temperature (°C)*	*R (μC/sec)*	*R (mC/day)*	*C* (μC/cm³)*
He	190	$0.73 \cdot 10^{-3}$	$6.31 \cdot 10^{-2}$	$0.93 \cdot 10^{-7}$
	243	$5.72 \cdot 10^{-3}$	$4.94 \cdot 10^{-1}$	$7.26 \cdot 10^{-7}$
	290	$2.38 \cdot 10^{-2}$	2.05	$3.02 \cdot 10^{-7}$
Ar–5% CH_4	190	$6.10 \cdot 10^{-4}$	$5.27 \cdot 10^{-2}$	$7.75 \cdot 10^{-8}$
	240	$5.20 \cdot 10^{-3}$	$4.49 \cdot 10^{-1}$	$6.60 \cdot 10^{-7}$
	290	$2.60 \cdot 10^{-2}$	2.24	$3.30 \cdot 10^{-6}$
Ar–H_2O	240	$6.50 \cdot 10^{-3}$	$5.61 \cdot 10^{-1}$	$8.25 \cdot 10^{-7}$
	280	$2.20 \cdot 10^{-2}$	1.90	$2.79 \cdot 10^{-6}$
N_2	185	$4.22 \cdot 10^{-4}$	$3.64 \cdot 10^{-2}$	$5.35 \cdot 10^{-8}$
	240	$5.93 \cdot 10^{-3}$	$5.12 \cdot 10^{-1}$	$7.55 \cdot 10^{-7}$
	285	$4.49 \cdot 10^{-2}$	3.87	$5.70 \cdot 10^{-6}$
Air	240	$3.63 \cdot 10^{-3}$	$3.13 \cdot 10^{-1}$	$4.60 \cdot 10^{-7}$
	285	$6.50 \cdot 10^{-2}$	5.61	$8.25 \cdot 10^{-6}$
H_2	148	$4.57 \cdot 10^{-4}$	$3.94 \cdot 10^{-2}$	$5.80 \cdot 10^{-8}$
	195	$1.05 \cdot 10^{-2}$	$9.00 \cdot 10^{-1}$	$1.33 \cdot 10^{-6}$
	250	$4.62 \cdot 10^{-2}$	3.99	$5.85 \cdot 10^{-6}$
	290	$4.29 \cdot 10^{-2}$	3.70	$5.35 \cdot 10^{-6}$

* For molecular tritium the occupation maximum permissible concentration in air is $2 \cdot 10^{-3}$ μC/cm.³.

TABLE II

ESTIMATED HALF LIFE OF TRITIUM FOIL FOR VARIOUS GASES AT THE TEMPERATURES INDICATED

Gas	*Temperature (°C)*	*Half life calculated* (days)*
He	190	547
	243	71.2
	290	16.9
Ar–5% CH_4	190	650
	240	72.6
	290	14.7
Ar–H_2O	240	61.5
	280	18.2
N_2	185	960
	240	67.6
	285	8.96
Air	240	109
	285	6.16
H_2	148	887
	195	38.4
	250	8.64
	290	9.35

* Half life calculated on basis of simple first order kinetics.

indicate the decrease in activity due to normal radioactive decay. This decrease in activity due to radioactive decay will not be of the same order of magnitude as the loss rate at elevated temperatures and therefore may be disregarded. Calculated half life due to leakage ranges from 960 days at 185° to 6.16 days at 285°, again depending upon the gas present. A slight discrepancy in the data for hydrogen is noted between 250° and 290°. This may be accounted for by considering the rapid decrease in source activity at these elevated temperatures. From the calculated half life values one would predict that the observed saturation current of a detection device should decrease to half of its original value in one half life of the associated radioactive foil. In practice this has not always found to be true. This apparent contradiction can best be explained by considering the characteristics of each individual source, which can reasonably be expected to vary from one source to another.

CONCLUSIONS

The data obtained in this investigation provide a guide for the selection of operating parameters when using tritium foils. Minor variations might occur for a given source, and indeed, variations would be expected to occur between different sources. The general observations, however, should remain valid.

The generally accepted maximum operating temperature for tritium foils is around 225°. The results herein reported indicate that when hydrogen is used as the carrier gas leakage losses become apparent at a much lower temperature than with the other gases tested. For molecular tritium, the occupation maximum permissible concentration in air is $2 \cdot 10^{-3}$ μC/cm^3 (ref. 2). Thus, if the ventilation conditions for a given site are known, the foregoing guide may be used to determine approximate concentration levels. As suggested by WILSON, it is advisable to lead the outlet of the detection device containing the radioactive foil into an extract or a fume hood if operation at elevated temperature is contemplated.

ACKNOWLEDGEMENT

The authors wish to express their sincere thanks to Tracerlab, Inc., Houston, Texas for the preparation of radioactive foils used in these experiments and for the use of their facilities.

SUMMARY

By means of a simple ionization chamber the loss rate of tritium from tritium impregnated foils was determined. An average 50 mC source was investigated, the loss rate being determined with respect to carrier gas and temperature. The maximum temperature suggested for the use of these foils has been 225° due to health hazards involved. Our results confirm these figures as the maximum allowable concentration in air is not exceeded at this temperature even under the most adverse conditions. From a practical viewpoint, however, it may be desirable to operate somewhat below this temperature in order to obtain a reasonable lifetime of the source in certain carrier gases.

REFERENCES

[1] F. VANDERSCHMIDT, J. R. ROEHRIG AND J. C. SIMONS, JR., *Use of Tritium as an Ionizing Source in Instruments*, presented at the Nuclear Engineering and Science Conference, Chicago, Illinois, March 17, 1958.
[2] E. J. WILSON, *Tritium Sources for Use in Chromatography Detectors*, Radiochemical Centre Information Sheet, Oct. 1, 1961.
[3] M. P. TAYLOR, *J. Chromatog.*, 9 (1962) 28.
[4] R. T. OVERMAN AND H. M. CLARK, *Radioisotope Techniques*, McGraw-Hill, New York, 1960.
[5] *Radioisotope Experiments for the Chemistry Curriculum*, manual prepared for the United States Atomic Energy Commission by Nuclear of Chicago.
[6] J. SHARPE, *Nuclear Radiation Detectors*, John Wiley & Sons, New York, 1955.

SEPARATION OF ISOMERIC LONG-CHAIN POLYHYDROXY ACIDS BY THIN-LAYER CHROMATOGRAPHY

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The resolution of higher fatty acid isomers and vinylogues by thin-layer chromatography (TLC) on adsorbents impregnated with complexing agents has recently been reported[1]. It was demonstrated that *threo* and *erythro* isomers of vicinal dihydroxy acids could be separated by TLC on silicic acid impregnated with boric acid. Boric acid or borates are known to form complexes more readily with *threo* glycols than with *erythro* glycols[2,3] and it is assumed that some such interaction was operative, under the conditions of chromatography, to result in the clear resolutions of dihydroxy isomers obtained.

These results were similar to those obtained by FRAHN AND MILLS by paper electrophoresis[3] and suggested the possibility that TLC on adsorbents impregnated with complexing agents might be of more general application to the resolution of certain isomeric compounds, particularly where lack of solubility in suitable electrolytes precludes the use of the electrophoretic method. It was therefore considered worthwhile to study the migrations of other polyhydroxy acids on thin-layer plates impregnated with boric acid and with other known glycol-complexing agents.

Some remarkable separations of isomeric polyhydroxy fatty acid esters were achieved and are described in this communication. The chemical and stereochemical factors which gave rise to these separations are not discussed in detail, since they have not yet been fully elucidated. These aspects of this work are currently being studied and will be reported in detail in a subsequent publication.

EXPERIMENTAL

Materials

The *erythro* and *threo* isomers of 6,7- and 9,10-dihydroxystearic acids were prepared by oxidation of the *cis* and *trans* forms of the corresponding olefinic acids with dilute alkaline permanganate[4]. The *erythro*- and *threo*-12,13-dihydroxyoleic and -stearic acids were derived from *cis*-12:13-epoxyoleic acid, isolated from *Vernonia anthelmintica* seed oil[5]. The trihydroxy- and tetrahydroxystearic acids were prepared, as described previously, from ricinoleic acid[6], from 9-hydroxyoctadec-12-enoic acid isolated from *Strophantus* seed oils[7], and from *cis*-12:13-epoxyoleic acid[5]. Each hydroxylation reaction produced a pair of isomeric tri- or tetrahydroxy acids which were separated as their methyl esters by preparative TLC on Silica Gel G, except for the *erythro*- and

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threo-9,12,13-trihydroxystearic acid pairs which were not resolved for this work. Methyl esters were prepared by adding excess diazomethane in diethyl ether to methanol solutions of the acids.

Procedures

Thin layers (*ca.* 275 μ) of Merck "Silica Gel G"* were applied to glass plates (20 × 20 cm or 10 × 20 cm) with the Desaga equipment**, as described by MANGOLD[8]. Impregnation of the layers was carried out by using aqueous solutions of the relevant compounds, instead of water, to prepare the adsorbent slurry for spreading on the plates. As a standard procedure, 2.8 g of impregnating agent was dissolved in 50 ml of water and mixed with 25 g of Silica Gel G, so that uniform impregnation of 10 % (w/w) was achieved. Silica gel layers were impregnated with the following compounds: boric acid, sodium borate, sodium arsenite, basic lead acetate, sodium metavanadate and sodium molybdate.

Alternatively, impregnation was achieved by spraying silica gel plates with *ca.* 20 % solutions of the inorganic compounds *cf.* ref. 1. However, this latter procedure gave less uniform impregnation which resulted, in some cases, in rather different migration patterns for some groups of isomers, so that layers impregnated during preparation were preferred for all comparative work. All plates, whether impregnated or not, were activated by heating in an oven at 110° for 30 min just before use.

Samples were applied as dilute solutions in chloroform and plates were developed, under conditions of "tank saturation", in closed jars lined with solvent-soaked filter paper. Chloroform–methanol mixtures were used as developing solvents. Visualisation of separated components was achieved generally by charring at 200° after spraying with 50 % aqueous sulphuric acid, or by viewing under ultraviolet light after spraying with a 0.2 % ethanolic solution of 2',7'-dichlorofluorescein.

RESULTS

The chromatograms of the methyl esters of long-chain di-, tri- and tetrahydroxy acids on untreated Silica Gel G (A), boric acid-impregnated Silica Gel G (B), sodium borate-impregnated Silica Gel G (C), and sodium arsenite-impregnated Silica Gel G (D) are reproduced in Figs. 1, 2 and 3.

It was found that Silica Gel G layers impregnated with basic lead acetate, sodium metavanadate, or sodium molybdate resulted in migrations of all components almost identical to those obtained on untreated Silica Gel G.

Dihydroxy esters

On untreated Silica Gel G, there is a slight gradation in mobility of dihydroxy esters according to the position of the glycol group in the chain, the polarity sequence being 6,7- > 9,10- > 12,13-dihydroxystearate > 12,13-dihydroxyoleate (Fig. 1A). There is, however, no difference in mobility according to *erythro*- or *threo*-configuration of the glycol group.

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* E. Merck, A.-G., Darmstadt, Germany; British distributor: Anderman and Co. Ltd., Tooley Street, London, S.E. 1.

** Desaga, G.m.b.H., Heidelberg, Germany; British distributor: Camlab (Glass) Ltd., Cambridge.

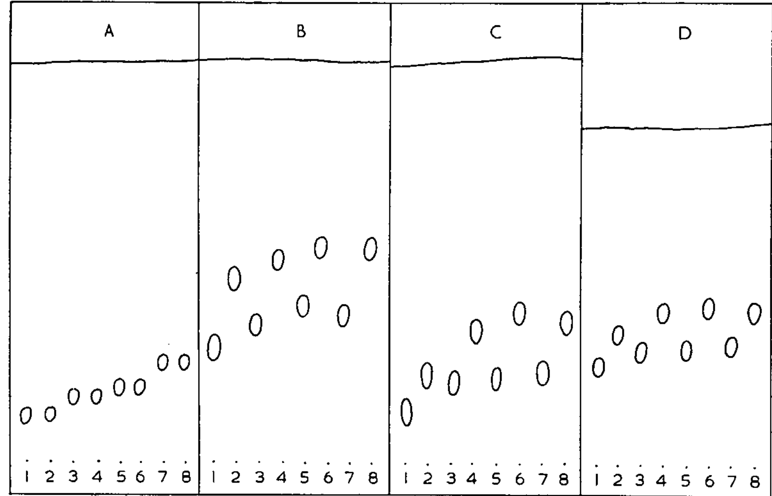

Fig. 1. Thin-layer chromatograms, on Silica Gel G (A), boric acid-impregnated Silica Gel G (B), sodium borate-impregnated Silica Gel G (C), and sodium arsenite-impregnated Silica Gel G (D), of methyl esters of the following fatty acids: 1 = *erythro*-6,7-dihydroxystearic; 2 = *threo*-6,7-dihydroxystearic; 3 = *erythro*-9,10-dihydroxystearic; 4 = *threo*-9,10-dihydroxystearic; 5 = *erythro*-12,13-dihydroxystearic; 6 = *threo*-12,13-dihydroxystearic; 7 = *erythro*-12,13-dihydroxyoleic; 8 = *threo*-12,13-dihydroxyoleic. Developing solvent: methanol–chloroform (2:98). Spots were located by spraying with aqueous sulphuric acid (1:1) and charring, and were reproduced by tracing.

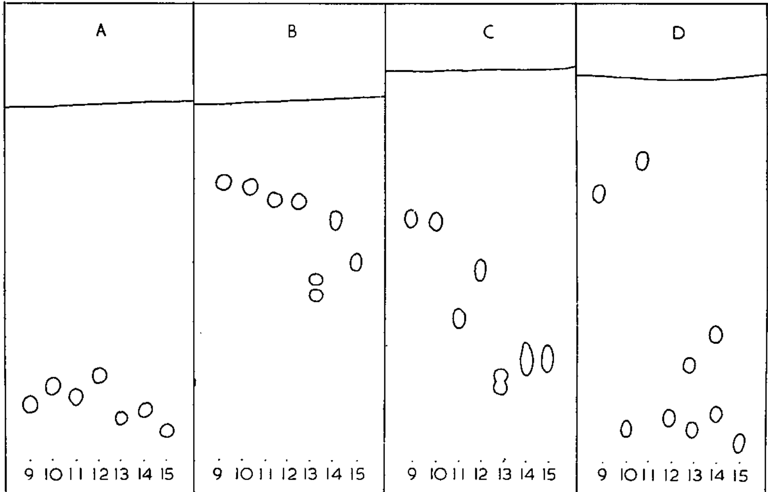

Fig. 2. Thin-layer chromatograms, on Silica Gel G (A), boric acid-impregnated Silica Gel G (B), sodium borate-impregnated Silica Gel G (C), and sodium arsenite-impregnated Silica Gel G (D), of methyl esters of the following fatty acids (literature melting points of acids in parentheses): 9 = *erythro*-9,10,12-trihydroxystearic (138°); 10 = *erythro*-9,10,12-trihydroxystearic (112°); 11 = *threo*-9,10,12-trihydroxystearic (110°); 12 = *threo*-9,10,12-trihydroxystearic (87°); 13 = *erythro*-9,12,13-trihydroxystearic isomers (148° and 102°); 14 = *threo*-9,12,13-trihydroxystearic isomers (89° and ?); 15 = *threo*-9,10,16-trihydroxypalmitic. Developing solvents: A, B and C, methanol–chloroform (5:95); D, methanol–chloroform (1:99). Spots were located by spraying with aqueous sulphuric acid (1:1) and charring, and were reproduced by tracing.

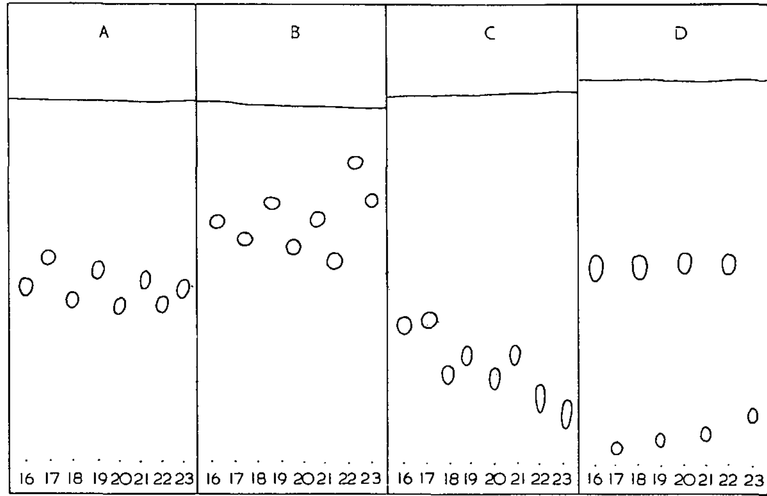

Fig. 3. Thin-layer chromatograms, on Silica Gel G (A), boric acid-impregnated Silica Gel G (B), sodium borate-impregnated Silica Gel G (C), and sodium arsenite-impregnated Silica Gel G (D), of methyl esters of the following tetrahydroxystearic acids (literature melting points of acids in parentheses): 16 = *erythro*-9,10-*erythro*-12,13- (177°); 17 = *erythro*-9,10-*erythro*-12,13- (156°); 18 = *threo*-9,10-*erythro*-12,13- (156°); 19 = *threo*-9,10-*erythro*-12,13- (130°); 20 = *erythro*-9,10-*threo*-12,13- (165°); 21 = *erythro*-9,10-*threo*-12,13- (112°); 22 = *threo*-9,10-*threo*-12,13- (148°); 23 = *threo*-9,10-*threo*-12,13- (122°). Developing solvents: A, B and C, methanol–chloroform (10:90); D, methanol–chloroform (4:96). Spots were located by spraying with aqueous sulphuric acid (1:1) and charring, and were reproduced by tracing.

On layers of Silica Gel G, impregnated with boric acid (Fig. 1B), sodium borate (1C), or sodium arsenite (1D), a clear separation of each diastereoisomeric pair is readily achieved. The lower melting *threo*-isomer of each pair has migrated faster on these impregnated layers.

Trihydroxy esters

The trihydroxy esters studied, like the dihydroxy esters, show little or no differences in rates of migration on untreated Silica Gel G that could be attributed to *erythro*- or *threo*-configuration of their glycol grouping. The 9,10,12-trihydroxystearates (9–12), however, do show a segregation of the diastereoisomers of each oxidation pair. The higher melting isomer (9 and 11), in each case, is more polar than the lower melting form (10 and 12). This resolution was utilised, in the preparation of these compounds for this work, to isolate the individual isomers from the pair of products resulting from hydroxylation. The 9,12,13-trihydroxystearate pairs (13,14) are not separable, in this way, into their high and low melting, disastereoisomeric forms. As expected, 9,10,16-trihydroxypalmitate (15) is more polar than the other trihydroxy esters studied, due to its shorter chain length and the fact that one of its hydroxyl groups is primary.

Boric acid-impregnated Silica Gel G gives a somewhat different pattern of migration of the trihydroxy esters (Fig. 2B). All compounds have migrated rather further than on untreated Silica Gel G with the same solvent system. This was also shown with the di- and tetrahydroxy esters and indicates that modification of the layer by incorporating boric acid in the adsorbent reduces its adsorbability. There is,

however, no obvious pattern of separation as a function of *threo-* or *erythro*-configuration of the glycol grouping, as was the case with the dihydroxy esters. Such an effect seems to be shown by samples 13,14 and 15, where the *threo*-9,12,13-trihydroxystearate pair and the *threo*-9,10,16-trihydroxypalmitate have migrated somewhat faster than and are clearly resolved from the *erythro*-9,12,13-trihydroxystearate pair. This last named pair of diastereoisomers (15) has been resolved into individual isomer spots although the *threo*-pair (14) has not. The 9,10,12-trihydroxystearates, however, show no similar separation according to *threo-* or *erythro*-configuration, the *threo*-compounds in fact being slightly more polar than their *erythro*-isomers. Also, the greater polarity of the high melting isomer of each pair has been lost and these compounds (9 and 11), are, if anything, slightly less polar on this medium than their lower melting isomers (10 and 12).

Sodium borate impregnation gives an even less predictable pattern with the trihydroxy esters (Fig. 2C). The *threo*-9,12,13-trihydroxystearate pair (14) and *threo*-9,10,16-trihydroxypalmitate (15) are only slightly less polar than the *erythro*-9,12,13-trihydroxy pair, which in this case also shows some resolution into individual isomers. The *threo*-9,10,12-trihydroxystearates (11 and 12), on the other hand, are held back appreciably, relative to their *erythro*-analogues (9 and 10), which is the reverse effect to that shown with the dihydroxy esters on the same adsorbent.

The separations of the trihydroxy isomers obtained on sodium arsenite-impregnated Silica Gel G (Fig. 2D) are more dramatic and potentially more useful. The higher melting isomer of each 9,10,12-trihydroxystearate pair (9 and 11) has migrated very much faster than the corresponding lower melting form (10 and 12). The *erythro*- and *threo*-9,12,13-trihydroxystearate pairs (13 and 14) have also been clearly resolved. The higher melting *erythro*-9,12,13-trihydroxy isomer was again the least polar and in the *threo*-9,12,13-trihydroxy pair the lower spot represents the ester of the acid melting at 89°, the melting point of the acid isomeric to this one is as yet undetermined[7]. In addition to these "major" separations there has also been shown some differentiation between comparable analogues (*e.g.* 9 and 11, 10 and 12) as a function of the *erythro-* or *threo*-configuration of their glycol group. The *threo*-isomer has in each case migrated slightly further than the *erythro*-isomer, as was the case with the dihydroxy esters. These two types of differences in migration are sufficient to allow complete resolution of all four diastereoisomers of 9,10,12-trihydroxystearate and of 9,12,13-trihydroxystearate on sodium arsenite-impregnated silica gel.

Tetrahydroxy esters

On untreated Silica Gel G (Fig. 3A), the 9,10,12,13-tetrahydroxystearates behave similarly to the 9,10,12-trihydroxy compounds. No differentiation has occurred on the basis of *threo-* or *erythro*-configurations of glycol groups but the higher melting isomer of each oxidation pair is somewhat more polar than the lower melting form. This difference in mobility was again utilised for the isolation of the individual isomers from these oxidation pairs, by preparative thin-layer chromatography.

A reversal of mobilities of the isomers in each oxidation pair is the result of impregnating the Silica Gel G with boric acid (Fig. 3B) and the higher melting form migrates the faster in each case. There is again no obvious separation as a result of the *erythro-* or *threo*-configurations of the glycol groups, although the di-*threo*-isomers (22 and 23) are somewhat less polar than the other six isomers.

On sodium borate-impregnated layers a further difference in migrating pattern is evident (Fig. 3C). The tetrahydroxystearates here show a pattern which could be attributed, at least in part, to the configurations of the glycol groups, but this has operated in the reverse direction to that found with the dihydroxy esters on the arsenite-impregnated layer. Thus the two di-*erythro*-compounds (16, 17) are least polar, the four compounds having one *erythro*- and one *threo*-glycol group (18–21) are more polar, and the two di-*threo*-compounds (22 and 23) are more polar still. The relative positions of the two isomers of each oxidation pair is also rather anomalous. The *threo-erythro* pair and the *erythro-threo* pair have maintained the same pattern as on untreated silica gel, with the higher melting form being more polar, but this pattern has been largely lost with the di-*erythro*-pair and has been inverted with the di-*threo*-pair.

As with the trihydroxy esters, the separation pattern of the tetrahydroxystearates on sodium arsenite-impregnated Silica Gel G (Fig. 3D) is remarkable but consistent. The higher melting isomer of each oxidation pair has migrated much faster and, with the more polar, low melting isomers at least, there is a subsidiary separation effect which could be attributed to *threo*- or *erythro*-conformations of glycol groups, di-*threo*- (23) migrating faster than *threo-erythro*-, or *erythro-threo*-, (19, 21) and these in turn migrating faster than the di-*erythro*-isomer (17).

DISCUSSION

The results described above demonstrate that the migration characteristics of polyhydroxy compounds on thin-layer chromatograms can be markedly altered by incorporating various inorganic materials in the adsorbent layer. These changes in migration characteristics are such that clear separation of several diastereoisomeric compounds, on the basis of *threo* or *erythro* configuration of glycol groups or of other stereochemical differences between isomers, has been achieved.

threo- and *erythro*-Dihydroxy isomers can be readily differentiated, on the micro scale, by TLC on Silica Gel G impregnated with boric acid, sodium borate or sodium arsenite. The separation of oxidation pairs of tri- and tetrahydroxystearates is possible, in some cases, on untreated, boric acid-impregnated or sodium borate-impregnated Silica Gel G layers and, in all cases studied, on sodium arsenite-impregnated layers. These separations may also be carried out on the preparative scale so that the tedious fractional crystallisation procedures required for separation of such isomers[5–7], may be obviated. By suitable combination of these chromatographic methods, the identity of a micro-sample of any one of the tri- or tetrahydroxy esters described in this work could be positively determined, if the whole series were available for comparison.

Insufficient is known, as yet, about the type of interaction between migrating substances and impregnated adsorbent, to enable valid conclusions to be drawn as to the relative conformations of diastereoisomers separated in this way. The use of thin-layers impregnated with glycol-complexing agents has been shown to result in changes in migration characteristics and consequent resolution of some sugars[9,10] and of some phenol-carboxylic acids (1-carboxy-3,4-dihydroxybenzene derivatives)[11]. In this last work, several impregnating agents were used and it was assumed that chelate formation occurred and was responsible for the observed changes in migration

patterns. Although this assumption may well be correct, not enough is yet known about this type of interaction on thin-layer chromatograms to allow its unqualified acceptance as the basis of detailed stereochemical interpretation of the results described in this paper. A purely physical interaction between active sites introduced into the layer by impregnation and preferred conformations of the hydroxy groups in the polyhydroxy ester isomers could conceivably result in the migration patterns achieved. In this connection, it should be noted that the resolution of the oxidation pairs of 9,10,12-tri- and 9,10,12,13-tetrahydroxystearates on untreated Silica Gel G must be due to some such purely physical interaction, since chemical chelating is clearly not possible in this case.

It is, in fact, considered that some form of chemical chelating with the impregnating agents is at least one factor, and probably the main factor, in producing the observed migration patterns. Several lines of evidence suggest that this is so, including the qualitative similarity of some the of results to those obtained in the electrophoretic system of FRAHN AND MILLS[3], where chemical chelating of glycols with borate was demonstrably the factor causing separation. Also, in the present work, it was found that sodium borate-impregnated Alumina G layers afforded a similar degree of separation, with the same solvent system, of the dihydroxy esters (1–8) as did borate-impregnated Silica Gel G, but in the reverse direction; *i.e.* the *threo*-dihydroxy esters were held back relative to the *erythro*-isomers. The only relevant difference in conditions is that the Silica Gel G layer has an acidic character whereas the Alumina G layer is basic. Thus, the observed reversal of pattern may be due either to (1) formation of boric acid esters on the acidic Silica Gel G layer and borate complexes on the basic Alumina G layer (*cf.* ref. 12), the different products then having lesser and greater polarities respectively than the unaffected *erythro*-isomers, or to (2) formation of borate complexes on both layers, which are then influenced by opposite ion-exchange effects on the acidic and basic layers. From consideration of other results, (2) seems more likely but either possiblity would indicate that chemical, rather than physical, interactions occur and are responsible for the separation of stereoisomers.

These conditions are, at present, of no great assistance in determining the relative configurations of the hydroxy groups of isomeric tri- and tetrahydroxystearates from the observed chromatographic behaviour. A detailed study of the migration characteristics of model dihydroxy compounds of known configurations, under the conditions described here and more strictly controlled conditions of pH, will be necessary before these phenomena may be understood sufficiently to be utilised for conformational analysis of more complicated molecules. This work is currently proceeding and will be reported in detail elsewhere. In the meantime, the practical utility of this method for separation of suitable diastereoisomeric polyhydroxy compounds has been amply demonstrated.

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SUMMARY

The migration characteristics, on silica gel thin-layer plates, of a series of dihydroxy, trihydroxy and tetrahydroxy long-chain fatty acid methyl esters have been examined. By incorporating various inorganic glycol-complexing agents in the adsorbent layers, considerable variations in migration characteristics were produced. Diastereoisomeric compounds were clearly resolved on the basis of *threo* or *erythro* configurations of glycol groups or of other stereochemical differences between such isomers.

REFERENCES

1 L. J. MORRIS, *Chem. Ind. (London)*, (1962) 1238.
2 H. WITTCOFF, O. A. MOE AND M. H. IWEN, *J. Am. Chem. Soc.*, 70 (1948) 742.
3 J. L. FRAHN AND J. A. MILLS, *Australian J. Chem.*, 12 (1959) 65.
4 A. LAPWORTH AND E. N. MOTTRAM, *J. Chem. Soc.*, (1925) 1628.
5 K. E. BHARUCHA AND F. D. GUNSTONE, *J. Chem. Soc.*, (1956) 1611.
6 J. P. KASS AND S. B. RADLOVE, *J. Am. Chem. Soc.*, 64 (1942) 2253.
7 F. D. GUNSTONE AND L. J. MORRIS, *J. Sci. Food Agr.*, 10 (1959) 522.
8 H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.
9 G. PASTUSKA, *Z. Anal. Chem.*, 179 (1961) 427.
10 V. PREY, H. BERBALK AND M. KAUSZ, *Mikrochim. Acta*, (1961) 968; (1962) 459.
11 J. HALMEKOSKI, *Suomen Kemistilehti*, 35 (1962) 39.
12 J. DALE, *J. Chem. Soc.*, (1961) 922.

EINE MULTIDIMENSIONALE TECHNIK ZUR CHROMATOGRAPHISCHEN IDENTIFIZIERUNG VON AMINOSÄUREN

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1. EINLEITUNG

Seit den grundlegenden Arbeiten von CONSDEN, GORDON UND MARTIN[1] ist die Trennung und Identifizierung von Aminosäuren durch zweidimensionale Papierchromatographie in zahlreichen Variationen immer wieder versucht worden (vgl. die übersichtlichen Darstellungen in Zit. 2–7). Verschiedene Autoren haben diese Methodik in den letzten Jahren auch für dünne Schichten von Silicagel modifiziert[8–11].

Mit zunehmender Anzahl der aus Naturstoffen isolierten "seltenen" Aminosäuren liess sich eine chromatographische Auftrennung und Identifizierung dieser Stoffe mit der Kombination von nur zwei Lösungsmittelsystemen und einigen Farbreaktionen längst nicht mehr bewältigen. Elution der in unverändertem Zustand relativ schwierig zu lokalisierenden Aminosäuren bzw. ihrer Gemische und ein- oder mehrmalige Rechromatographie in anderen Systemen einschliesslich Elektrophorese erwiesen sich als unbedingt notwendig.

Um diese zeitraubenden, stufenweisen Operationen wie zerstörungsfreie Lokalisierung, Elution und Rechromatographie vieler Einzeleluate zu vermeiden, haben wir eine Kombination von vier Lösungsmittelsystemen und verschiedenen Farbreaktionen zu einer neuartigen quasi-4-dimensionalen Technik entwickelt; sie hat sich in den letzten anderthalb Jahren sowohl nach der Filterpapier- wie auch Cellulose-Dünnschicht-Technik im praktischen Gebrauch gut bewährt.

2. PRINZIP DER METHODE

Das Prinzip dieser multidimensionalen Technik lässt sich einfach anhand des Beispiels in Fig. 1 erläutern.

In den Ecken von 3 Celluloseblättern oder Cellulose-Dünnschichtplatten (A, B, C) wird das zu trennende Gemisch wie üblich aufgetropft, in diesem Beispiel ein Gemisch von Hydroxyprolin (Hypro), Glycin (Gly), Lysin (Lys), Histidin (Hist), Tryptophan (Try) und Leucin (Leu)*. In der ersten Dimension (in Fig. 1 von oben nach unten) werden sowohl A wie B und C mit dem *gleichen* Lösungsmittelsystem I chromatographiert, wobei sich eine erste Auftrennung ergibt, z.B. in die Gruppen Hypro + Gly + Lys + His und Try + Leu, wie in Fig. 1 links aussen angedeutet.

* Für die Kurzbezeichnung der Aminosäuren folgen wir den Vorschlägen von BRAND[12]; siehe Tabelle I.

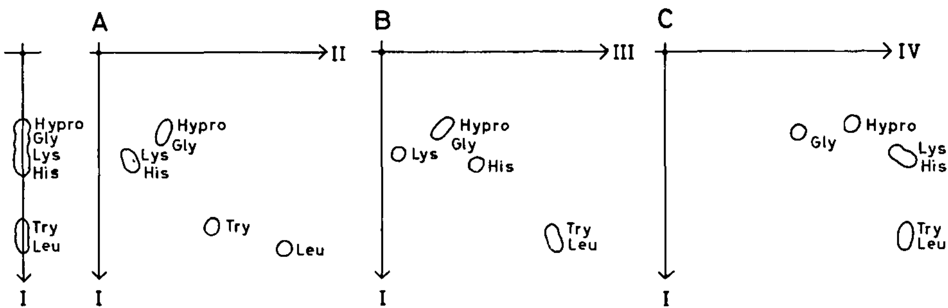


Fig. 1. Schema der multidimensionalen Technik für die Trennung und Zuordnung von Aminosäuren. Links aussen: Eindimensionales Chromatogramm mit System I allein. (A) zweidimensionales Chromatogramm, Lösungsmittelkombination: I + II; (B) zweidimensionales Chromatogramm, Lösungsmittelkombination: I + III; (C) zweidimensionales Chromatogramm, Lösungsmittelkombination: I + IV.

In der *zweiten* Dimension (in Fig. 1 von links nach rechts) wird nun jedes Chromatogramm für sich mit einem Lösungsmittelsystem *unterschiedlicher* Zusammensetzung entwickelt (II für A, III für B und IV für C). Auf diese Art erhält man drei 2-dimensionale Chromatogramme (I + II, I + III, I + IV), in welchen die Reihenfolge der Aminosäuren in der ersten Dimension die gleiche ist, während ihre Sequenz in den zweiten Dimensionen durch die Auswahl geeigneter Systeme (II, III, IV) so stark variiert wird (Inversionen), dass in Kombination mit 2–5 Farbreaktionen eine sichere *Zuordnung* und *Unterscheidung* von 52 der in Tabelle I erwähnten 57 Amino-

TABELLE I

QUALITATIVE UND QUANTITATIVE NACHWEISBARKEIT VON 57 AMINOSÄUREN (ALPHABETISCHE REIHENFOLGE)

(In μg/Fleck auf dem zweidimensionalen Chromatogramm der Kombination I + II). NC = Ninhydrin–Collidin-Reagens; I = Isatinreagens; PD = Pauly's Diazo Reagens; RH = Reindel-Hoppe-Modifikation; NPK = Nitroprussid-Natrium–Kaliumferricyanid. Die quantitativen Angaben gelten nur für NC, und zwar stellen sie den unteren praktischen Bereich der Nachweisbarkeit dar; die unterste Grenze liegt in der Regel noch tiefer, variiert aber etwas je nach den Trocknungs-Bedingungen nach der Applikation von NC; die Angaben über die Färbungen mit den Reagentien I, PD, RH und NPK entsprechen einem Einsatz von je 0.5–5 γ Aminosäuren. Bei PD bedeutet + eine gelbe bis rötliche Farbe, bei RH eine blauschwarze und bei NPK eine rote; (+) bedeutet eine nur schwache Farbreaktion.

Aminosäure	*Kurz-bezeichnung*	*NC*		*I*	*PD*	*RH*	*NPK*
α-Alanin	Ala	0.05	violett	violett		—	
β-Alanin	βAla	0.05	grün	lila		+	
α-Amino-*n*-buttersäure	Abu	0.05	violett	lila		(+)	
α-Aminoisobuttersäure	isoAbu	0.05	violett	gelb		(+)	
β-Aminobuttersäure	βAbu	0.05	lila	gelbl.		+	
β-Aminoisobuttersäure	βisoAbu	0.05	violett	lila		+	
γ-Aminobuttersäure	γAbu	0.2	violett	lila		+	
ε-Amino-*n*-capronsäure	εAcap	1.0	violett	rosa		+	
p-Aminohippursäure	pAhip	10.0	violett	gelb		+	
Arginin	Arg	0.2	violett	rosa		+	+
Asparagin	$Asp(NH)_2$	2.0	gelb	rosa		+	
Asparaginsäure	Asp	0.05	grün	violett		(+)	

(Forsetzung P. 331)

TABELLE I (*Forsetzung*)

Aminosäure	*Kurzbezeichnung*	*NC*		*I*	*PD*	*RH*	*NPK*
Canavaninsulfat	Can	0.2	violett	bräunl.		+	+
Citrullin	Cit	0.1	violett	rosa		+	
Cysteinsäure	$CySO_3H$	0.05	violett	gelb		(+)	
Cystin	$(Cys)_2$	1.0	braun	rosa		(gelb)	
α,γ-Diaminobuttersäure	Dabu	2.0	violett	rosa		+	
α,α-Diaminopimelinsäure	Dapim	2.0	violett	rot		(grau)	
Dihydroxyphenylalanin	Dopa	10.0	grau	lila	+	(gelb)	
Dijodtyrosin	DiJtyr	5.0	violett	lila	(+)	+	
Dimethylcystein	Dimecys	0.2	violett	rosa		—	
Glutamin	$Glu(NH_2)$	0.5	violett	rosa		(+)	
Glutaminsäure	Glu	0.05	violett	lila		—	
Glycin	Gly	0.05	braun	rosa		+	
Glycocyamin	Gcy	—	—	rosa		(+)	+
Histidin	His	0.3	grau	lila	+	(grau)	
Hydroxyglutaminsäure	Hyglu	0.05	violett	rosa		(+)	
Hydroxylysin	Hylys	0.5	violett	lila		+	
Hydroxyprolin	Hypro	1.0	gelb	blau		(+)	
β-Hydroxyvalin	Hyval	0.5	violett	rosa		(+)	
Isoleucin	Ileu	0.1	violett	rosa		(+)	
allo-Isoleucin	*allo*-Ileu	0.1	violett	rosa		(+)	
Kreatin	Kn	—	—	gelb	(+)	+	+
Kreatinin	Knin	—	—	gelb		+	+
Kynurenin	Kyn	0.5	braun	rosa		(gelb)	
Lanthionin	Lan	1.0	violett	orange		(rosa)	
Leucin	Leu	0.5	violett	rosa		—	
Lysin	Lys	0.5	violett	rot		+	
Methionin	Met	0.5	violett	rosa		—	
Methioninsulfon	$MetSO_2$	0.2	violett	rosa		(+)	
Methioninsulfoxyd	MetSO	0.2	violett	rosa		(braun)	
Norleucin	Nleu	0.5	violett	lila		—	
Norvalin	Nval	0.05	violett	rot		(+)	
Ornithin	Orn	0.2	violett	rot		+	
α-Phenylalanin	Phe	0.2	violett	lila		(gelb)	
α-Phenylglycin	Phegly	5.0	gelb	gelb	(+)	(braun)	
Prolin	Pro	0.5	gelb	blau		(+)	
Sarkosin	Sar	0.1	grau	gelb		+	
Serin	Ser	0.2	violett	orange		gelb	
Taurin	Taur	0.2	violett	gelb		+	
Threonin	Thr	0.5	violett	rosa		(+)	
allo-Threonin	*allo*Thr	0.5	violett	rosa		(+)	
Thyronin	Thyron	2.0	braun	braun		—	
Thyroxin	Thyrox	2.0	braun	gelb	+	(+)	
Tryptophan	Try	0.5	violett	lila		(braun)	
Tyrosin	Tyr	0.5	braun	rot	+	(rosa)	
Valin	Val	0.05	violett	rosa		(+)	

säuren möglich wird (Cystein wird hierbei als Cystin oder Cysteinsulfosäure chromatographiert). Dihydroxyphenylalanin (Dopa) verteilt sich auf eine so grosse Fläche (siehe Fig. 2–4), dass sich seine Identifizierung als schwierig erweist. Die restlichen vier Aminosäuren Leucin, Norleucin, Isoleucin und *allo*-Isoleucin trennen sich zwar von allen andern 53, untereinander aber nur in die Gruppen Leu + Nleu und Isoleu + *allo*-Isoleu auf. Während sich das Diastereomeren-Paar Threonin und *allo*-Threonin dank der stärkeren Wechselwirkung zwischen NH_2- und OH-Gruppen ohne weiteres nach obiger Technik trennen lässt, ist dies für Isoleu und *allo*-Isoleu chromato-

graphisch sehr schwierig (Wechselwirkung zwischen NH_2- und CH_3-Gruppen zu schwach). Leu und Nleu sind hingegen in einem Spezialsystem gut differenzierbar (System Nr. 22 von Tabelle II auf Cellulose-Dünnschicht, zweimaliger Lauf).

3. AUSWAHL DER SYSTEME UND DURCHFÜHRUNG DER METHODE

Die Methode wurde ursprünglich für die konventionelle Filterpapierchromatographie entwickelt und schliesslich unter Beibehaltung der Lösungsmittelsysteme auf die Cellulose-Dünnschichtchromatographie übertragen, wodurch in erster Linie eine 5–10-fach höhere Empfindlichkeit erreicht worden ist. Aus einer grösseren Anzahl bekannter oder neuer Lösungsmittelsysteme kamen nach eindimensionalem Lauf der verschiedensten Aminosäuren 28 Systeme (siehe Tabelle II) in die engere Auswahl;

TABELLE II

LÖSUNGSMITTELSYSTEME DER ENGEREN AUSWAHL FÜR DIE TRENNUNG VON AMINOSÄUREN AUF CELLULOSE (Nr. 1–28) UND SILICAGEL (Nr. 1–31)*

Nr.	System
1	*n*-Butanol–Äthanol–Wasser (10:10:5)
2	*n*-Propanol–Äthylacetat–Wasser (7:1:2)
3	*tert.*-Amylalkohol–Isopropanol–Wasser (100:40:55)
4	*tert.*-Amylalkohol–Dimethylformamid–Wasser (100:15:45)
5	Äthanol–8 %ig. wässr. NaCl (3:1)
6	*n*-Butanol–Essigsäure–Wasser (4:1:5)
7	*n*-Butanol–Essigsäure–Wasser (100:10) bis zur Sättigung (ca. 30)
8	Isopropanol–Ameisensäure–Wasser (40:2:10) = *System II*
9	*n*-Butanol–Ameisensäure–Wasser (480:6:314)
10	*n*-Propanol–Propionsäure–Wasser (70:5:20)
11	Isopropanol–Zitronensäure–Wasser (40:2:10)
12	*sec.*-Butanol–Isopropanol–Monochloressigsäure–Wasser (70:10:3 g:40)
13	Phenol–Wasser (75:25)
14	Phenol–Wasser (75:25) (+7.5 mg Na-cyanid) Sättigung der Gasphase mit 3 %ig. wässr. NH_4OH = *System IV*
15	Collidin–Lutidin–Wasser (1:1) ges.
16	Methyläthylketon–Pyridin–Wasser (60:15:25)
17	*n*-Butanol–Pyridin–Wasser (10:3:3)
18	Chloroform–Methanol–17 %ig. NH_4OH (20:20:9)
19	*sec.*-Butanol–3 %ig. wässr. NH_4OH (100:44)
20	*n*-Butanol–Äthanol–Diäthylamin–Wasser (10:10:2:5)
21	*n*-Butanol–Aceton–Diäthylamin–Wasser (10:10:2:5) = *System I*
22	*tert.*-Amylalkohol–Isopropanol–Triäthylamin–Veronal–Wasser (100:40:0.8:1.8 g:50)
23	*sec.*-Butanol–Methyläthylketon–Dicyclohexylamin–Wasser (10:10:2:5) = *System III*
24	*sec.*-Butanol–Äthanol–Dicycohexylamin–Wasser (100:25:15:35)
25	*n*-Propanol–Pyridin–Essigsäure–Wasser (15:10:3:12)
26	*sec.*-Butanol–Isopropanol–Wasser–Veronal-Na (5 %ig. in Wasser) (100:15:60:10)
27	*sec.*-Butanol–Isopropanol–Triäthylamin–Veronal–Wasser (100:10:0.8:1.8g:60)
28	*sec.*-Butanol–Isopropanol–Phosphatpuffer, pH 8–Wasser (35:35:10:25)
29	Benzol–Aceton–Methanol–Essigsäure (70:5:20:5)
30	Äthylacetat–Methyläthylketon–Ameisensäure–Wasser (5:3:1:1)
31	Äthylacetat–Pyridin–Wasser (20:10:11)

* Zum grössten Teil handelt es sich um publizierte Systeme (vgl. Zit. 2) oder Modifikationen davon.

von diesen wurden erst nach rechnerischer und schliesslich experimenteller Kombination von Systempaaren folgende vier Lösungsmittelsysteme in der gegebenen Reihenfolge ausgewählt.

System I (erste Dimension für A, B und C):

n-Butanol–Aceton–Diäthylamin–Wasser (10:10:2:5), nach HARDY *et al.*[13], pH 12.0 (auf Cellulose 11.05–11.4).

System II (zweite Dimension für A):

Isopropanol–Ameisensäure (99 %-ig)–Wasser (40:2:10), pH 2.5 (auf Cellulose 2.7–2.8).

System III (zweite Dimension für B):

sec.-Butanol–Methyläthylketon–Dicyclohexylamin–Wasser (10:10:2:5), modifiziert nach HARDY *et al.*[13], pH 11.0 (auf Cellulose 10.9–11.0).

System IV (zweite Dimension für C):

Phenol–Wasser (75:25), Gasphase equilibriert mit 3 %igem wässrigen NH_4OH, vgl. Zit. 2–7 (auf Cellulose pH 7.1–7.3).

Wie ersichtlich unterscheiden sich diese Systeme u.a. durch ihr pH und ihre Eigenschaft als Wasserstoff-Akzeptoren bzw.-Donatoren.

Die festgelegte Reihenfolge der Systeme richtete sich nicht nur nach der Güte der damit zu erzielenden Trennungen sondern auch nach Gesichtspunkten der Flüchtigkeit und der Verträglichkeit mit nachfolgenden Systemen und Farbreaktionen. Von letzteren verwendeten wir Ninhydrin–Collidin (NC)[2–7], Isatin (I)[14], Pauly's Diazo-Reagens (PD)[2–7], das modifizierte Reagens nach Reindel-Hoppe (RH)[15] und Nitroprussid-Natrium–Kaliumferricyanid (NPK)[3]; über deren Spezifität und Empfindlichkeit gibt Tabelle I Auskunft. Nach der Kombination I + II sind alle fünf Farbreaktionen anwendbar, nach I + III noch NC, I, PD und NPK (RH fällt aus), nach I + IV wegen der schwierig zu entfernenden Phenolreste noch NC, I und NPK (PD und RH fallen aus).

Als Cellulose-Träger dienten entweder Whatmanpapiere No. 1 oder dünne Schichten von Cellulosepulver Macherey und Nagel MN 300. Im folgenden geben wir unsere Präparation nach der Methode von STAHL[16] mit dem Applikator der Firma Desaga an.

8 g Cellulosepulver werden in 48 ml Wasser und 2 ml Äthanol suspendiert und 2–3 Min. mit einem elektrischen Mixer (Handmixer Arria, 1. Stufe, Teflonscheibe, 25 mm Durchmesser mit 3 Einschnitten) homogenisiert; der Brei wird bei 250 μ Schlitzbreite über 5 Glasplatten (20 × 20 cm) gestrichen; Trocknung über Nacht horizontal bei Raumtemperatur. Für das Phenol-System IV erweist sich eine Vorwaschung der Schicht mit System I aufsteigend und quer zur Streichrichtung (entsprechend der Laufrichtung der zweiten Dimension) als sehr vorteilhaft; Trocknung wie oben.

Bei Verwendung aller drei Lösungsmittelsysteme (II, III, IV) für die zweiten Dimensionen und nur einer Farbreaktion benötigt man drei Cellulose-Dünnschichtplatten (CP); da wir in der Regel noch drei weitere Farbreaktionen für die erste Kombination I + II vorsehen, geben wir den Arbeitsgang für total sechs CP wieder.

Man legt sechs CP nebeneinander, mit der Streichrichtung von oben nach unten und zeichnet die Startpunkte in der Ecke rechts unten je 25 mm vom Plattenrand entfernt oberflächlich an. Die wässrige Aminosäurelösung wird mit Mikropipette in Portionen von 1 μl oder weniger aufgetropft (Fleckendurchmesser 3 mm oder weniger) und mit kalter oder warmer Luft angetrocknet. Die Konzentration der einzelnen Aminosäuren soll 10 μg möglichst nicht überschreiten. Bei Gemischen mit sehr

grossem Konzentrationsunterschied der Komponenten sind notfalls Chromatogramme mit verschiedener Beladung auszuführen.

Für den ersten Lauf werden die sechs CP entweder gemeinsam in einem passenden Messingrahmen (Plattenabstand 5 mm, Schicht gegen Schicht) oder paarweise (je eine an der Wand, Schicht gegen innen) so in die Trennkammern gestellt, dass die Laufrichtung (aufsteigend) mit der Streichrichtung identisch ist. Die Trennkammern werden bereits vorher mit soviel Lösungsmittelsystem I gefüllt, dass der Abstand zwischen Flüssigkeitsniveau und Startpunkt 15–20 mm beträgt (ca. 150–200 ml pro Trennkammer je nach ihrer Form und Beladung); die Eintauchhöhe der CP selbst ist hierbei weitgehend belanglos. Wichtig ist, dass die Kammern vorher *nicht* mit Hilfe von Filtrierpapierstreifen oder sonstigen Massnahmen maximal mit Dampf gesättigt worden sind; eine gewisse *Untersättigung* ist für den guten Lauf Vorbedingung und kann leicht reproduziert werden, indem man das Lösungsmittel erst unmittelbar vor der Chromatographie einfüllt. Der Lauf (bei 22°) wird unterbrochen, sobald die Lösungsmittelfront das obere CP-Ende erreicht hat (Zeit siehe Tabelle III). Die CP

TABELLE III

VERGLEICH DER LAUFZEITEN (STUNDEN) DER EINZELNEN SYSTEME AUF VERSCHIEDENEN TRÄGERN ÜBER DIE ERFORDERLICHE DISTANZ UNTER VERWENDUNG DER GEGEBENEN EINRICHTUNGEN

	Whatman Nr. 1 (*absteigend, 35 cm*)	*CMN 300 (50 μ)* (*aufsteigend, 15 cm*)	*Kieselgel G (240 μ)* (*aufsteigend, 15 cm*)
System I	5	2.25	3
System II	9	3.5	5
System III	5.25	2	4
System IV	13.5 (aufsteigend)	4	5

werden in gleicher Stellung wie in den Kammern 5–10 Min. bei 90° (Trockenschrank) oder 12 Std. bei Raumtemperatur getrocknet. Nun tropft man ein Gemisch von Leitsubstanzen (0.5 μg Gly, 1 μg Tyr, 1 μg Nleu in total 1 μl) seitlich auf die Startlinie für den Lauf in der zweiten Dimension (identisch mit der Linie der Chromatographie in der ersten Dimension) und zwar so, dass es einerseits 5–10 mm links vom ursprünglichen Startpunkt anderseits in gleicher Richtung 20 mm vor der ersten Front (= Plattenrand) placiert ist (auf Fig. 2–4 als "Test" bezeichnet).

Nun werden die CP mit der neuen Startlinie nach unten (senkrecht zum ersten Lauf) in anderen Trennkammern in der zweiten Dimension chromatographiert: je zwei CP in zwei Kammern mit System II, eine CP in je eine Kammer mit System III und IV (Systeme frisch einfüllen, Untersättigung, Füllvolumen bzw. Abstand Niveau-Startlinie wie beim ersten Lauf). Nach aufsteigender Chromatographie bis zum oberen Rand werden die Platten 20 Min. bei 90° getrocknet.

Wird aus irgendwelchen Gründen die konventionelle Papierchromatographie vorgezogen, nimmt man drei Bögen Whatmanpapier No. 1, z.B. 45 × 45 cm, zeichnet den Startpunkt je 9 cm von den Rändern in einer Ecke an und chromatographiert nach Auftropfen im System I absteigend im Durchlauf so lange bis das als Leitfarbstoff dienende Kresolrot (unmittelbar neben dem Startpunkt aufgetropft) 80 % der

Bogenlänge zurückgelegt hat. Nach Trocknen chromatographiert man wie üblich senkrecht zur ersten Dimension: im System II absteigend bis das Kresolrot den unteren Papierrand erreicht, bzw. im System III absteigend bis Tartrazin als Leitfarbstoff noch 3 cm vom unteren Bogenrand entfernt ist, bzw. im System IV aufsteigend (technisch einfacher mit dem Phenol-System). Nach üblicher Trocknung sind die Chromatogramme bereit zur Behandlung mit Nachweisreagenzien.

Nachweis

(*a*) *Ninhydrin-Collidin-Reagens* (*NC*). 1 g Ninhydrin in 700 ml Äthanol abs., 29 ml 2,4,6-Collidin und 210 ml Essigsäure. Gleichmässig auf je eine CP der drei Kombinationen sprühen und trocknen bei 90° bis Flecke erscheinen (Untergrund bleibt noch weiss); die sofort sichtbaren Flecke werden zur Erleichterung der Auswertung mit weichem Bleistift markiert. Nach einigen Stunden bei Raumtemperatur werden sie bedeutend intensiver und "fliessen" eventuell zusammen, zum Teil (je nach Aminosäure und Konzentration) erscheinen sie erst dann deutlich.

(*b*) *Isatin* (*I*) *nach* BARROLIER *et al.*[14]. Stark sprühen und trocknen 30 Min., 90°; besser differenzierte Farben entstehen jedoch beim Trocknenlassen bei 20° (20 Std.).

(*c*) *Pauly's Diazo-Reagens* (*PD*). Lösung 1: 0.4 *M* Natriumsulfanilat in Wasser; Lösung 2: 0.4 *M* Natriumnitrit in Wasser. Vor Gebrauch mischt man 1 Vol. Lösung 1, 1 Vol. Lösung 2, 8 Vol. 0.25 *N* Salzsäure und 10 Vol. 2 *N* Sodalösung und besprüht die Chromatogramme.

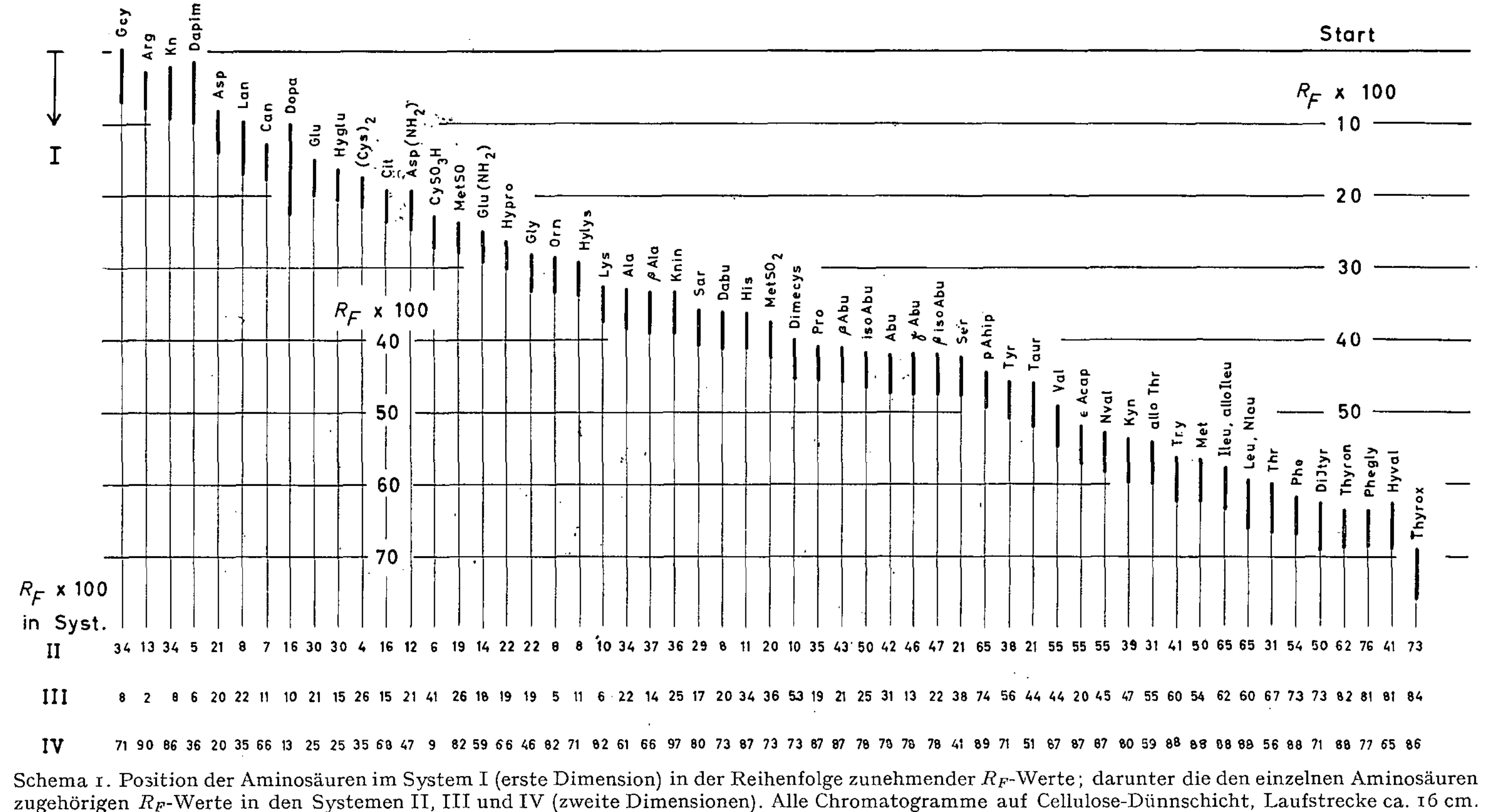
(*d*) *Reindel-Hoppe-Reagens* (*RH*), modifiziert nach GREIG UND LEABACK[15]. Lösung 1: 2 %ige wässrige Kaliumhypochloritlösung; Lösung 2: gleiche Volumina einer gesättigten Lösung von *o*-Tolidin in 2 %iger Essigsäure und einer 0.85 %igen wässrigen Lösung von Kaliumjodid werden vor Gebrauch gemischt. Die Chromatogramme werden leicht mit Lösung 1 besprüht und 1–1.5 Std. bei Raumtemperatur stehen gelassen (gewisse Lösungsmitteldämpfe beeinträchtigen die Chlorierung!). Darauf mit Lösung 2 gleichmässig besprühen: schwarzblaue Flecken auf weissem Untergrund.

(*e*) *Nitroprussid-Natrium-Kaliumferricyanid* (*NPK*) nach Vorschrift D 106 in Zit. 3: Je 1 Vol. 10 %ige Natronlauge, 10 %ige Nitroprussid-Natriumlösung und 10 %ige Kaliumferricyanidlösung werden mit 3 Vol. Wasser gemischt; nach 0.5 stündigem Stehenlassen sprühen.

4. AUSWERTUNG

Wir beschreiben die Auswertung anhand der Cellulose-Dünnschicht-Chromatogramme.

In Schema 1 sind die Aminosäuren in der Reihenfolge ihrer zunehmenden R_F-Werte im System 1 (erste Dimension) angeordnet wobei ihre R_F-Bereiche durch einen fetten senkrechten Strich gekennzeichnet sind. Darunter, mit den einzelnen Aminosäuren durch feine Striche verbunden, finden sich die zugehörigen R_F-Werte in den drei für die zweiten Dimensionen verwendeten Systemen II, III und IV, und zwar bereits unter Berücksichtigung allfälliger leichter Verdrängungseffekte wenn alle Aminosäuren zugleich chromatographiert werden. *Dadurch ist die Position für jede Aminosäure in den drei zweidimensionalen Chromatogrammen eindeutig fixiert*: Unsicherheiten in der Zuordnung sind durch die erzielten Unterschiede und Inversionen in den zweiten Dimensionen bei gleichbleibender Sequenz in der ersten Dimension so gut wie ausgeschlossen. Voraussetzung dazu ist selbstverständlich eine gute



Schema 1. Position der Aminosäuren im System I (erste Dimension) in der Reihenfolge zunehmender R_F-Werte; darunter die den einzelnen Aminosäuren zugehörigen R_F-Werte in den Systemen II, III und IV (zweite Dimensionen). Alle Chromatogramme auf Cellulose-Dünnschicht, Laufstrecke ca. 16 cm.

Reproduzierbarkeit, zu deren Realisierung die methodischen Einzelheiten von grosser Wichtigkeit sind (siehe oben unter Ausführung und unten unter Diskussion). Trotzdem lässt sich eine gesamthafte mässige Verschiebung der für die Systeme II, III und IV angegebenen R_F-Werte nach oben oder unten nicht immer vermeiden. Eine einfache Kontrolle der Reproduzierbarkeit und Zuordnung lässt sich dadurch erreichen, dass man jeweils *nach* der ersten Dimension zu beiden Seiten der neuen Startlinie für die zweite Dimension ein Gemisch von Gly, Try und Nleu auftropft, welche dann nach Sichtbarmachung des fertigen zweidimensionalen Chromatogrammes als Leitsubstanzen für die Zuordnung dienen (gelegentliche Verdrängungseffekte auf dem zweidimensionalen Chromatogramm sind hierbei zu berücksichtigen); dies ist insbesondere dann von Vorteil, wenn keine Aminosäuren vorliegen, die sich durch eine besondere charakteristische Färbung hervorheben. Letztere ist ein weiteres, wertvolles Hilfsmittel für die Zuordnung und damit Identifizierung, weshalb die Kombination I + II von Anfang an 3-fach ausgeführt wird. Je ein Chromatogramm der Kombination I + II, I + III und I + IV wird mit Ninhydrin–Collidin entwickelt, je eines der Kombination I + II zusätzlich mit Diazo-Reagens und Isatin oder gegebenenfalls mit weiteren Spezialindikatoren. Die qualitativen und quantitativen Aspekte der Farbreaktionen sind aus Tabelle I ersichtlich. Bei der Chromatographie aller erwähnten Aminosäuren erhält man Flecken-Muster wie sie aus Fig. 2–4 ersichtlich sind. Es handelt sich um Photographien von Originalchromatogrammen auf denen die Flecke (auch die hierbei weniger gut reproduzierbaren) leicht angezeichnet und beschriftet worden sind.

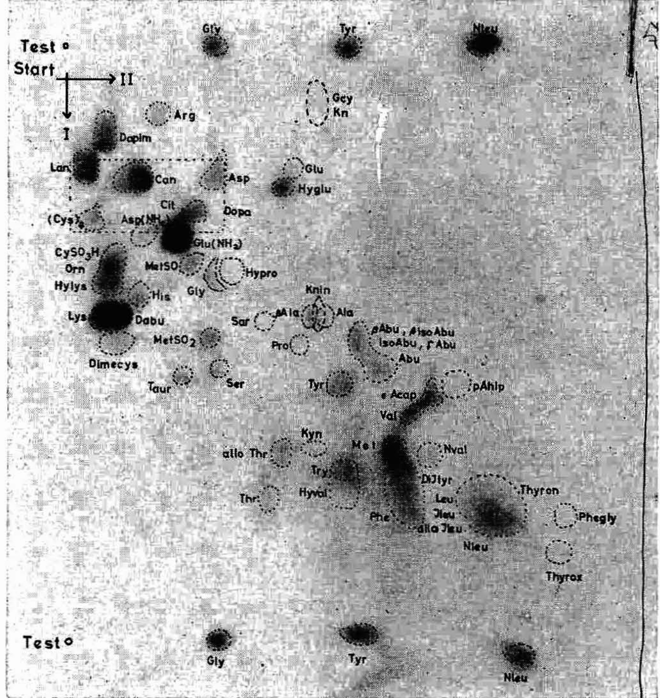


Fig. 2.

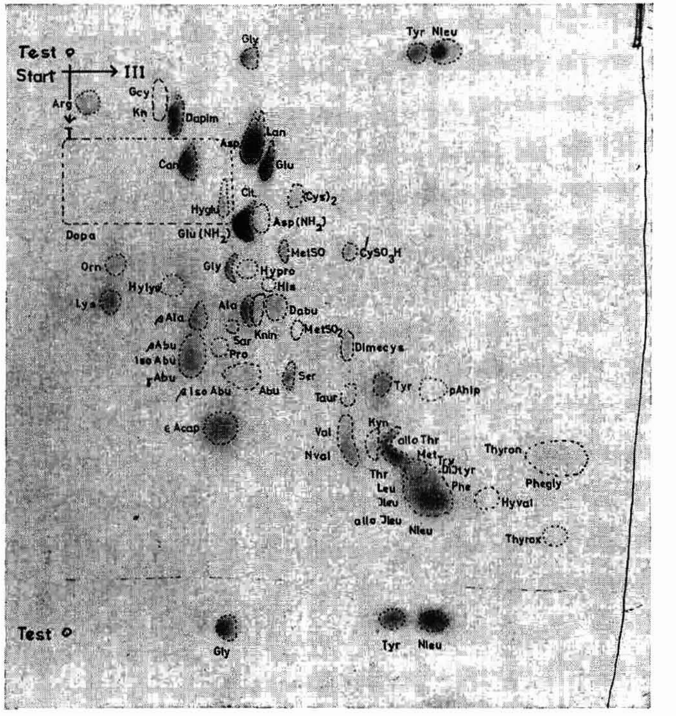


Fig. 3.

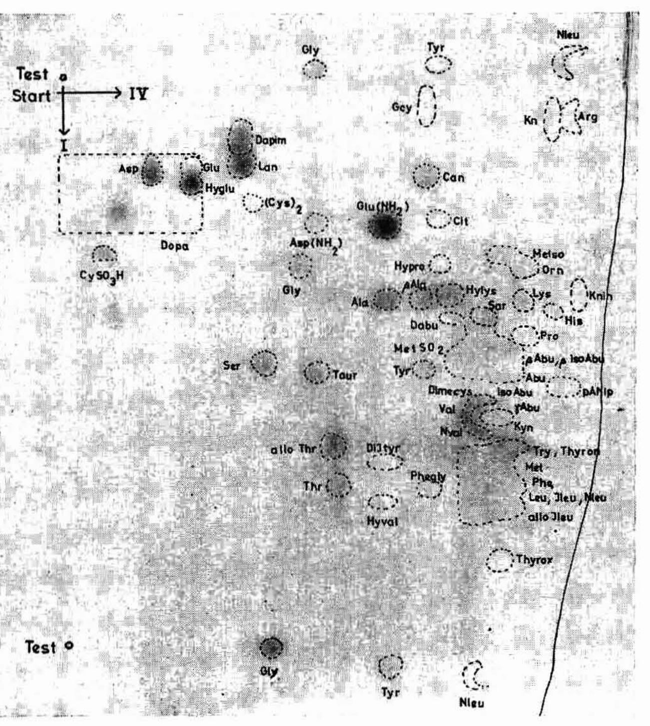


Fig. 4.

Fig. 2–4. Photographien der 3 zweidimensionalen Cellulose-Dünnschicht-Chromatogramme von 57 Aminosäuren mit den Lösungsmittel-Kombinationen I + II, I + III und I + IV. Da ein Teil der Flecke (z.B. die gelben) photographisch schlecht reproduzierbar ist, wurden alle zur Verdeutlichung eingeringelt und ausserdem zugeordnet (Abkürzungen siehe Tabelle I). Die Konzentration der einzelnen Aminosäuren betrug ca. die 2–6 fache Menge der in Tabelle I angegebenen Minimalkonzentrationen. Die grössere markierte Fläche entspricht der diffusen Verteilung des schwer löslichen Dopa. Nicht eingeringelte Flecke oder dünklere Partien entsprechen nicht näher definierten Verunreinigungen bzw. Untergrundfärbung. Für die Ausführung siehe Text; Platten-Dimension 20 × 20 cm, Frontlinie markiert.

Die Zuordnung der einzelnen Flecken zu den Aminosäuren erfolgt z.B. so, dass man alle drei Chromatogramme, mit dem ursprünglichen Startpunkt links oben, nebeneinander auflegt (vgl. in Fig. 2–4). Zweckmässigerweise bedient man sich eines Lineales über alle drei Platten und beginnt mit der Zuordnung reihenweise von oben nach unten mit Hilfe der Fleckenkarten (Fig 2–4) oder des Schema 1; anderseits kann man auch bei einem Fleck mit prägnanter Färbung oder Position beginnen. Ausser zur Identifizierung unbekannter Aminosäureflecken lässt sich Schema 1 auch zur Auswahl der besten Kombinationen für die Auftrennung vorbekannter Amino säuregemische heranziehen; oft werden für diese Zwecke 1–2 zweidimensionale Chromatogramme genügen; als Beispiel sei die Analyse der Hydrolysenprodukte synthetischer Peptide angeführt, sei es zur qualitativen Kontrolle des Aminosäuregehaltes, sei es zur Untersuchung von Hydrolysenmethoden. In diesem Zusammenhang ist zu erwähnen, dass Di- und Oligopeptide in den hier verwendeten Systemen stets kleinere R_F-Werte aufweisen als ihre Bausteine.

5. DISKUSSION

In der Regel ziehen wir die Dünnschichttechnik für unser Verfahren vor, da sie geringere Laufzeiten (siehe Tabelle III) und vor allem 5–10-fach geringere Substanzmengen erfordert als die konventionelle Papierchromatographie; bei letzterer fällt dafür die Plattenbereitung weg und die Reproduzierbarkeit verlangt keine so strenge Standardisierung.

Die Wahl des geeigneten *Trägermaterials* für die Dünnschicht richtete sich sowohl nach Trenneffekt wie Nachweisempfindlichkeit. Bei Verwendung der gleichen Lösungsmittelsysteme (I–IV) mit Silicagel als Träger (Kieselgel G Merck) war der Trenneffekt ganz ungenügend; einerseits verursachten die basischen Systeme auf Silicagel besonders häufig störende Doppelfronten, anderseits durchschnittlich weniger ausgeprägte Inversionen als auf Cellulose. Wir versuchten daher, aus einer engeren Auswahl von ca. 30 Lösungsmittelsystemen (siehe Tabelle II), darunter auch die von BRENNER *et al.*[10,11] angegebenen, auf die gleiche Art wie vorher für Cellulose solche auszuwählen, die nach der quasi-4-dimensionalen Technik auch auf Silicagel eine vollständige Auftrennung der 53 Aminosäuren ermöglichen sollte. Als optimale Kombinationen der Systeme (siehe Tabelle II) fanden wir die Paare Nr. 18 + 30, 18 + 12 und 18 + 14. So konnten wir zwar mit Nr. 18 + 14 z.B. das Gemisch von Fig. 1 bereits völlig auftrennen, bei der Chromatographie der übrigen Aminosäuren ergaben sich jedoch immer wieder zahlreiche Paare oder sogar Tripletts, die sich nicht auftrennen liessen. Wir verzichteten daher auf Silicagel als Träger zugunsten von Cellulosepulver, obwohl die Nachweisempfindlichkeit auf ersterem für einige Aminosäuren noch etwas höher liegt als auf letzterem.

Von allen geprüften Cellulosequalitäten gab das Pulver MN 300 die schönsten Schichten und Chromatogramme, und zwar nach Homogenisation wie oben beschrieben; für den Trenneffekt war es belanglos ob die Streichdicke 250 oder 500 μ gewählt wurde; bei einer solchen von 250 μ mass die getrocknete Celluloseschicht nur 50 μ. Unhomogenisiertes Cellulosepulver hatte Flecke mit wellenförmigen Rändern zur Folge. Aufsprühen statt Streichen der Suspension erwies sich technisch als schwer durchführbar (Verstopfung). Eine gute *Reproduzierbarkeit* der Chromatogramme ist ausser von der Cellulosequalität, Schichtpräparation, Lösungsmittelalter, Trock-

nung zwischen beiden Läufen, usw. noch ganz besonders von der Kammersättigung abhängig. Maximale Kammersättigung verursacht zu raschen Lauf des Lösungsmittels bei aufsteigender Chromatographie so dass die Aminosäuren nur ungenügend aufgetrennt werden und zusammengeschoben erscheinen. Bei maximaler Sättigung dürfte nur im Durchlaufverfahren mit den angegebenen Lösungsmittelsystemen eine genügende Trennung zu erreichen sein. Es ist daher wichtig, dass bei unserem aufsteigenden Verfahren eine *Untersättigung* gewährleistet ist, wie im Abschnitt 3 beschrieben; dies trifft inbesondere für die Läufe in der zweiten Dimension zu.

Die Empfindlichkeit des *Nachweises* der Aminosäuren mit Ninhydrin erwies sich als stark abhängig von (a) Trägermaterial, (b) Lösungsmittelsystem, (c) den Aminosäuren selbst und (d) der Reagenszusammensetzung.

So ist das Ninhydrin–Essigsäure-Reagens (NE)* für Silicagelschichten, das Ninhydrin–Collidin-Reagens (NC) aber für Celluloseschichten am empfindlichsten; ein Vergleich ist daher nur unter Berücksichtigung dieser optimalen Bedingungen sinnvoll; er ergab eine ähnliche Empfindlichkeit für beide Trägersysteme mit Ausnahme einiger Aminosäuren wie, Alanin, Ornithin und Arginin, die auf Silicagel mit NE noch besser nachweisbar sind als auf Cellulose mit NC.

DANK

Frau D. HODEL-V. WESTERNHAGEN danken wir für ihre wertvolle und unermüdliche technische Hilfe bei der Ausführung der Chromatogramme.

ZUSAMMENFASSUNG

Für die chromatographische Differenzierung und Charakterisierung von 52 Aminosäuren wurde eine Kombination von vier Lösungsmittelsystemen und verschiedenen Farbreaktionen zu einer neuartigen multi-dimensionalen Technik entwickelt. Sie beruht auf drei zwei-dimensionalen Cellulose-Dünnschicht-Chromatogrammen, wobei für die erste Dimension immer das gleiche Lösungsmittelsystem, für die zweite Dimension 3 verschiedene Systeme geeigneter Zusammensetzung verwendet werden. Dadurch ist die Position jeder Aminosäure eindeutig fixiert. Die Vor- und Nachteile von Cellulose und Silicagel als Träger werden hinsichtlich Trennbarkeit, Reproduzierbarkeit und Nachweisempfindlichkeit des Verfahrens diskutiert.

SUMMARY

A novel multidimensional technique has been elaborated and found suitable for the chromatographic identification of 52 amino acids. This technique is based on three two-dimensional cellulose thin-layer chromatograms using the same solvent system for the first dimension and three different systems of suitable properties for the second dimensions. With this method all amino acids can be differentiated and characterized by fixed positions, and in addition by some colour reactions. The relative merits of cellulose and silicagel as carriers are discussed in relation to separation efficiency, reproducibility and sensitivity of detection.

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* Reagens Nr. 108, zitiert von M. BRENNER, A. NIEDERWIESER UND G. PATAKI in Zit. 16.

LITERATUR

[1] R. CONSDEN, A. H. GORDON UND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
[2] F. CRAMER, *Papierchromatographie*, 4. Aufl., Verlag Chemie, Weinheim/Bergstr., 1958.
[3] I. M. HAIS UND K. MACEK, *Handbuch der Papierchromatographie*, Bd. 1, Fischer Verlag, Jena, 1958.
[4] R. J. BLOCK, E. L. DURRUM UND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New York, 1958.
[5] I. SMITH, *Chromatographic Techniques*, Vol. 1, Heinemann–Interscience, London–New York, 1958.
[6] H. F. LINSKENS, *Papierchromatographie in der Botanik*, 2. Aufl., Springer, Berlin, 1959.
[7] E. LEDERER (Éditeur), *Chromatographie en Chimie Organique et Biologique*, Vol. 2, Masson, Paris, 1960.
[8] E. NÜRNBERG, *Arch. Pharm.*, 292/64 (1959) 610.
[9] E. MUTSCHLER UND H. ROCHELMEYER, *Arch. Pharm.*, 292/64 (1959) 449.
[10] M. BRENNER UND A. NIEDERWIESER, *Experientia*, 16 (1960) 378.
[11] A. R. FAHMY, A. NIEDERWIESER, G. PATAKI UND M. BRENNER, *Helv. Chim. Acta*, 44 (1961) 2022.
[12] E. BRAND, *Ann. N.Y. Acad. Sci.*, 47 (1946) 187.
[13] T. L. HARDY, D. O. HOLLAND UND J. H. C. NAYLER, *Anal. Chem.*, 27 (1955) 971.
[14] J. BARROLIER, J. HEILMAN UND E. WATZKE, *Z. Physiol. Chem.*, 304 (1956) 21.
[15] C. G. GREIG UND D. H. LEABACK, *Nature*, 188 (1960) 310.
[16] E. STAHL, *Dünnschichtchromatographie*, Springer, Berlin, 1962.

PYRENDERIVATE ALS FLUORESZENZINDIKATOREN BEI DER DÜNNSCHICHTCHROMATOGRAPHIE

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Bei der Anwendung der Dünnschichtchromatographie (D.C.) für präparative Trennungen und quantitative Bestimmungen muss man die Substanzen nach ihrer Trennung auf der Platte lokalisieren, ohne sie in der chemischen Struktur zu verändern. Die hierfür meist angewandten Methoden (z.B. des Anfärbens von Streifen auf dem Chromatogramm; siehe Zusammenstellungen in Zit. 1) sind recht umständlich und mit Substanzverlusten verbunden. Am günstigsten erschienen Verfahren, bei denen die Substanzen durch einen Fluoreszenzindikator lokalisiert werden. Von einem solchen Fluoreszenzindikator sollte man verlangen, dass er möglichst Substanzen jeglicher Struktur empfindlich nachzuweisen gestattet und dass er bei der Elution vom Adsorbens selbst nicht mit eluiert wird. Ferner sollte er nach Möglichkeit wasserlöslich und hitzebeständig sein, damit man ihn gleich bei der Herstellung der Platte auf das Adsorbens aufbringen kann. Man könnte dadurch das Ansprühen der Platte vermeiden, das für Fluoreszenzindikatoren ausserordentlich gleichmässig geschehen muss.

Die bisher benutzten Fluoreszenzindikatoren (anorganische Leuchtphosphore, Rhodamin B, 2′,7′-Dichlorfluorescein, Na-fluorescein, Bromthymolblau u.a.[1] erfüllen diese Forderungen nicht. Die besonders günstig erscheinenden anorganischen Leuchtphosphore ermöglichen nur bei im U.V.-Licht absorbierenden Substanzen einen guten Nachweis, während Substanzen ohne Absorption im U.V.-Bereich nur in höheren Konzentrationen, wenn überhaupt, nachgewiesen werden können. Lediglich Morin ist für Platten mit Al_2O_3 gut brauchbar[2]. Für das in der D.C. meist benutzte Kieselgel dagegen ist es viel weniger günstig.

Auf der Suche nach einem möglichst universell anwendbaren Fluoreszenzindikator untersuchten wir einige als optische Aufheller (Blankophore) technisch benutzte Substanzen*. Bessere Ergebnisse erzielten wir mit den Pyrenderivaten 3-Hydroxypyren-5,8,10-trisulfonsaures Natrium (P_0) und 3,5-Dihydroxypyren-8,10-disulfonsaures Natrium (P_2)[4]**. Zur Prüfung der Empfindlichkeit beim Nachweis verschiedener Substanzen in der D.C. wurde bei der Bereitung der Platten eine wässrige Lösung von P_0 oder P_2 (enthaltend $^1/_3$ mg P_0 bzw. $^1/_4$ mg P_2 auf je 1 g Kieselgel G)

* Wie wir nach Abschluss unserer Versuche feststellten, ist in der Literatur[3] die Anwendung eines optischen Aufhellers (Ultraphor WT, BASF) beim dünnschichtchromatographischen Nachweis von Weichmachern erwähnt. Es wird jedoch weder auf die allgemeine Anwendbarkeit des Nachweises noch auf die Verwendbarkeit in der präparativen D.C. hingewiesen.

** Die Substanzen werden von der Fa. Bayer, Leverkusen, hergestellt. Wir danken der Firma, insbesondere Herrn Prof. Dr. S. PETERSEN, für die grosszügige Überlassung dieser Pyrenderivate sowie von optischen Aufhellern.

der Streichmasse zugesetzt, und es wurden die Platten wie üblich beschichtet. Nach Aktivieren während 30 Min. bei 120° wurden die Platten im Exsikkator aufbewahrt. Die Substanzen müssen nach Möglichkeit in Chloroform, Essigester oder Aceton aufgebracht werden, da Methanol Startflecke zeigte. Nach Entwickeln mit Lösungsmittelgemischen, die R_F-Werte von etwa 0.3 bis 0.6 ergaben, trocknete man die Platten kurz bei 110° und betrachtete die Platten nach 5 Min. Liegenlassen an der Luft unter der U.V.-Lampe (~ 365 mμ). Die Substanzen wiesen sich auf schwach fluoreszierendem Untergrund je nach der chemischen Struktur durch helle Fluoreszenzen oder dunkle Löschungen aus. Fig. 1 zeigt ein Dünnschichtchromatogramm mit P_2 als Fluoreszenzindikator, das im U.V.-Licht photographiert wurde.

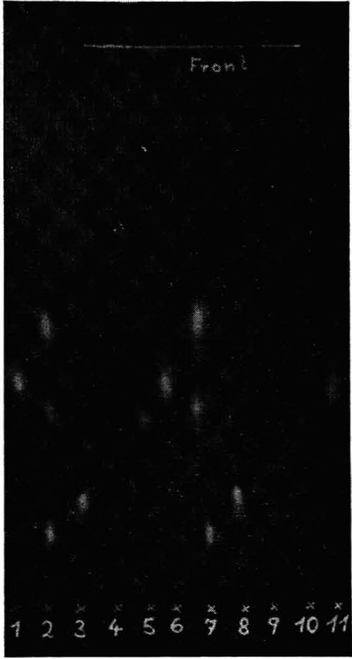

Fig. 1. Photographie eines Dünnschichtchromatogramms mit P_2 als Fluoreszenzindikator, aufgenommen im U.V.-Licht von 365 mμ auf Agfa AGP-Film. Chromatogramm entwickelt mit Benzol–Aceton (20:3); Adsorbens: Kieselgel H. Folgende Substanzen wurden aufgetragen: Auf Startpunkt 1, 6 und 11 Cholesterin (0.8γ, 1.2γ und 1.6γ); auf 2 und 7 eine Mischung von Cholestan-3β,5α,6β-triol-3,6-diacetat, Cholestan-3β,5α,6β-triol-3-methyläther-6-acetat und Cholestan-3β,5α,6β-triol-3-methyläther (je 0.5γ und 0.75γ); auf 3 und 8 das Diol Nr. 28 der Tabelle I (8γ und 12γ), auf 4 und 9 Progesteron (1γ und 1.5γ) und auf 5 und 10 Pentaacetyl-D-glucose (5γ und 7.5γ).

Beim Lagern der Platten im Exsikkator waren die Flecke nach einer Woche kaum verblasst. Über die Empfindlichkeit des Nachweises und die auftretenden Löschungen bzw. Fluoreszenzen gibt Tabelle I Aufschluss.

Für analytische Zwecke ist P_0 besser geeignet, da es einen empfindlicheren Nachweis der meisten Substanzen erlaubt (etwa um den Faktor 2–5), doch muss P_0 als technisches Material für präparative Trennungen durch Umfällen aus wenig Wasser–Äthanol gereinigt werden, um fluoreszierende und leicht auswaschbare Verunreinigungen zu entfernen. Es wird aber auch dann leichter von Kieselgel

TABELLE I

NACHWEIS VERSCHIEDENER SUBSTANZEN IN DER DÜNNSCHICHTCHROMATOGRAPHIE MIT 3,5-DIHYDROXYPYREN-8,10-DISULFONSAUREM NATRIUM

Nr.	*Substanz*	*Nachweis**		*Nr.*	*Substanz*	*Nachweis**	
1	Naphthalin	F	+	23	α-Chlordan	F	++
2	Anthracen	L	0.1 γ	24	β-Chlordan	F	++
3	Diels-Kohlenwasserstoff	F	++	25	Heptachlor	F	++
4	α-Äthylnaphthalin	F	+	26	Gammexan	F	++
5	Diphenyl	F	+	27	Hexachlorbenzol	F	++
6	Cholesterin	F	0.1 γ				
7	5β-Cholestan-3β-ol-methyläther	F	0.08 γ	28	(OH, OH, Cl_6)	F	1 γ
8	5α-Pregnan-3β,17α-diol	F	+++				
9	Progesteron	L	0.1 γ				
10	Dehydro-isoandrosteron	F	+++				
11	5β-Androstan-3α-ol-17-on	F	+++	29	(O, OH, OH, O, Cl_6)	F	++
12	digitogenindiacetat	F	+++				
13	Digoxigenin	F	+++				
14	Conessin	F**	+				
15	Funtumidin	F**	++	30	(O, O, Cl_6)	F	++
16	Brucin	F**	+				
17	Tryptamin	F**	+	31	α-Pentacetyl-D-glucose	F	1 γ
18	Veronal	F	+	32	2,3,4,6-Tetramethyl-D-glucose	F	20 γ
19	Aspirin	L	+				
20	Pyramidon	F	++	33	2,3,4-Trimethyl-D-galaktose	F	4 γ
21	Aldrin	F	1 γ				
22	Dieldrin	F	++	34	4,6-Dimethyl-D-glucose'	F	1 γ

* Auftretende Löschung (L) oder Fluoreszenz (F). Nachweisgrenze etwa: + = 2 – 5 γ; ++ = 0.2–1 γ; +++ = 0.01–0.1 γ; die in der Tabelle angegebenen Zahlenwerte sind die ermittelten unteren Nachweisgrenzen.

** Bei basischen Lösungsmittelsystemen fluoresziert der Untergrund gelbbraun, die Substanzen sind als helle Flecke zu erkennen.

eluiert als P_2. Es zeigt sich jedoch, dass die untersuchten Substanzen in den meisten Fällen auch mit P_2 noch unterhalb 1 γ gut nachgewiesen werden können. Tetramethylglucose (Nr. 32), die nur in höheren Konzentrationen (20 γ) zu erkennen ist, fällt etwas heraus, während Tri- und Dimethylzucker wieder sehr viel besser sichtbar zu machen sind.

Besonders empfindlich lassen sich Steroide der verschiedensten Struktur nachweisen. Günstig ist auch der Nachweis einer ganzen Reihe von Insektiziden (Nr. 21–30), die auf anderem Wege[5,6] nur recht umständlich und z.T. mit sehr viel geringerer Empfindlichkeit nachgewiesen werden können. So ist das Diol (Nr. 28) in einer Menge von 50 γ mit Rhodamin B[5] nicht mehr sicher feststellbar[7], während mit P_0 noch 0.8 γ erkannt werden können. Auch auf Platten mit Al_2O_3 als Adsorbens lassen sich Substanzen durch P_0 und P_2 sichtbar machen. Der Untergrund fluoresziert in diesem Falle gelbgrün.

Die Pyrenderivate P_0 und P_2 sind daher dank ihrer einfachen Anwendung und der Empfindlichkeit des durch sie ermöglichten Nachweises für analytische Chromatogramme ausgezeichnet geeignet. Man kann beim Mehrfachentwickeln und bei der zweidimensionalen Dünnschichtchromatographie nach jeder Entwicklung direkt das Trennergebnis begutachten. Der weitere Nachweis der Substanzen auf der Platte mit den üblichen Sprühmitteln wird nicht gestört, lediglich im U.V.-Licht ist ein

stärker fluoreszierender Untergrund vorhanden. Mit Lösungsmittelsystemen, die stärker polar als Methanol–Chloroform (7:3) sind, werden die Pyrenderivate teilweise ausgewaschen. In solchen Fällen werden sie besser nicht mit der Sorptionsschicht aufgebracht, sondern nach dem Entwickeln in einer Lösung in Aceton aufgesprüht.

Der besondere Vorteil bei der Anwendung der Pyrenderivate als Fluoreszenzindikatoren liegt aber in der präparativen Dünnschichtchromatographie, da durch sie die Substanzen, ohne dass sie chemisch verändert werden, zu erkennen sind und P_2 selbst durch Chloroform–Methanol (1:1) in der Siedehitze nicht vom Kieselgel eluiert wird.

EXPERIMENTELLES

Die Ausführung einer präparativen Dünnschichtchromatographie unter Verwendung von Pyrenderivaten sei am Beispiel der Trennung eines Gemisches von Cholestan-3β,-5α,6β-triol-3,6-diacetat, Cholestan-3β,5α,6β-triol-3-methyläther-6-acetat und Cholestan-3β,5α,6β-triol-3-methyläther beschrieben.

Eine Glasplatte (20 × 20 cm) wurde manuell mit einem homogenen Brei aus 15 g Kieselgel H (Merck), 2 mg P_2 und 44 ml Wasser gleichmässig beschichtet. Nach dreistündigem Trocknen an der Luft (die Platte liegt auf einer völlig ebenen Unterlage) wurde 1 Stunde bei 110° aktiviert. Das Substanzgemisch in gleichen Teilen (90 mg), in etwas Chloroform gelöst, wurde mittels einer Kapillare auf der Startlinie der Platte aufgetragen und in einem abgeschlossenen Chromatographiegefäss mit 100 ml Chloroform–Aceton (10:1.5) chromatographiert (Dauer: 1 Stunde). Auf der trockenen Platte liessen sich die drei Substanzen unter der U.V.-Lampe als 1 bis 1.5 cm breite fluoreszierende Zonen mit den R_F-Werten (gemessen von der Startlinie bis zur Mitte der Substanzzone) 0.75, 0.60 und 0.25 leicht erkennen. Das Kieselgel der markierten Zonen wurde mit dem Spatel von der Platte abgekratzt und mit je 30 ml Chloroform–Methanol (1:1) bei Zimmertemperatur und in der Siedehitze eluiert. Nach Abdestillieren des Lösungsmittels wurden 29 mg Diacetat, 29 mg Ätheracetat und 28 mg Äther erhalten. Die Substanzen waren dünnschichtchromatographisch völlig rein und fluoreszierten nicht im U.V.-Licht. Beim Vermessen ihrer U.V.-Absorption wurde ausschliesslich Eigenabsorption festgestellt, d.h. sie waren frei von Spuren des im U.V. stark absorbierenden Pyrenderivats.

In Mengen bis zu 1 g (je nach Zahl, Grösse und Schichtdicke der Chromatographieplatten* *zeichnet sich diese Art der präparativen Dünnschichtchromatographie gegenüber der Säulenchromatographie durch saubere Trennungen, Zeitgewinn und geringe Substanzverluste aus.* Da aufgrund dieser Vorteile die Säulenchromatographie im Bereich obiger Substanzmengen immer mehr von der präparativen Dünnschichtchromatographie abgelöst wird, ist die Anwendung der Pyrenderivate als Indikatoren besonders zu empfehlen.

DANK

Wir danken den Herren Dr. LUDWIG und Dr. VOGEL aus unserem Institut für die Überlassung der Substanzen Nr. 21–25 und Nr. 28–30 sowie Herrn STROH für die eifrige Mitarbeit bei der Ausführung der Versuche.

* Nach einem besonderen Verfahren von H. HALPAAP[8], ist es möglich, auf einer Platte von 1 m Breite und 2 mm Schichtdicke bis zu 10 g Substanzgemisch aufzutrennen.

ZUSAMMENFASSUNG

Für die analytische wie präparative Dünnschichtchromatographie wird ein Zusatz von geringen Mengen der Natrium-Salze von 3-Hydroxypyren-5,8,10-trisulfosäure oder der 3,5-Dihydroxypyren-8,10-disulfosäure zum Kieselgel empfohlen.

SUMMARY

For purposes of identification in analytical and preparative thin-layer chromatography, it is recommended to add small amounts of the sodium salt of 3-hydroxypyrene-5,8,10-trisulphonic acid or 3,5-dihydroxypyrene-8,10-disulphonic acid to the silica gel.

LITERATUR

[1] E. STAHL, *Dünnschichtchromatographie*, Springer Verlag, Berlin, 1962;
K. RANDERATH, *Dünnschichtchromatographie*, Verlag Chemie, Weinheim/Bergstr., 1962.
[2] V. CERNY, J. JOSKA UND L. LÁBLER, *Collection Czech. Chem. Commun.*, 26 (1961) 1658.
[3] J. W. COPIUS PEEREBOOM, *J. Chromatog.*, 4 (1960) 323.
[4] E. TIETZE UND O. BAYER, *Ann. Chem.*, 540 (1939) 189.
[5] D. WALDI, in E. STAHL, *Dünnschichtchromatographie*, Springer Verlag, Berlin, 1962, S. 374.
[6] H. J. PETROWITZ, *Chemiker-Ztg.*, 85 (1961) 867.
[7] Dr. G. LUDWIG, Bonn, Privatmitteilung.
[8] H. HALPAAP, *Chem. Ing. Technik*, 35 (1963) 486.

THE USE OF REPEATED DEVELOPMENT IN SPREAD-LAYER CHROMATOGRAPHY

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In paper partition chromatography, repeated one-dimensional development of the chromatogram with the same or different solvents is a current technique, and has frequently been utilized[1–4], *e.g.*, in the separation of steroids. To calculate the resulting R_F values after the second development, PRUSÍKOVÁ[3] derived a general relationship, which was verified practically[3–5]. Recently, thin-layer chromatography, *i.e.* chromatography on a thin non-adhering layer (thin-layer chromatography without binder, spread-layer chromatography), employing the technique of MOTTIER AND POTTERAT[6], has been successfully applied to the chromatographic separation of steroids[7–11]. The present study was undertaken to determine the value of the repeated development in this technique and to establish the factors which influence the mobility of the substances.

METHODS

Chromatographic procedure

Approximately 25 g alumina (activity II–III; 250 mesh) are spread on a glass plate (12 × 27 cm) and a layer 10 cm wide is smoothed by means of a glass rod which is sleeved at each end with a 2 cm length of polyethylene tubing. The thickness of the tubing is 0.6–0.8 mm and this determines the depth of the adsorption layer of alumina on the glass plate. Steroid samples or azodyes in chloroform are spotted approx. 2 cm from the narrower edge of the prepared glass plate.

The glass plate with the adsorption layer is placed in a glass tank (20 × 30 × 20 cm) at a slope of 15–25° from the horizontal and 50 ml of the mobile phase is poured into the bottom of the tank. The chromatogram is developed by the ascending technique until the solvent reaches the other side of the glass plate (approx. 30–45 min).

Detection

17-Ketosteroids were detected by spraying with the Zimmermann reagent (2 parts 1 % ethanolic *m*-dinitrobenzene, 1 part 5 *N* aqueous KOH) on the alumina surface when dry. The steroids appear as violet spots without heating after 2–3 min. Care must be taken not to disturb the alumina layer when spraying.

RESULTS AND DISCUSSION

Factors influencing the R_F values in a single run

The flow rate of the solvent front, *i.e.* the relation between the time and the distance travelled by the front from the start is parabolic, the first 5 cm being run within

5–10 min, depending on the nature of the mobile phase and other conditions, whereas 20 cm requires 30–60 min. The flow rate decreases with increasing slope of the glass plate and with decreasing grain size of alumina.

TABLE I

THE INFLUENCE OF GRAIN SIZE OF ALUMINA ON THE R_F VALUES OF 17-KETOSTEROIDS

Steroids	R_F	
	grain size	
	< 0.06 mm	*> 0.07 mm*
Androstane-3,17-dione	0.83	0.58
4-Androstene-3,17-dione	0.73	0.48
5-Androsten-3β-ol-17-one	0.41	0.26
Etiocholan-3α-ol-11,17-dione	0.28	0.18
5-Andrósten-3β-ol-7,17-dione	0.23	0.17
5-Androstene-3β,7α-diol-17-one	0.12	0.07
Flow rate of solvent front	22 cm/61 min	22 cm/23 min

Therefore, the R_F values, in the early stages, depend to a considerable degree on the distance the solvent has run and only become constant after a run of more than 10–12 cm. The slope of the glass plate and the distance of the starting line from the level of the solvent are of practically no importance. The R_F values increase with decreasing grain size of the adsorbent (Table I) and are influenced by the thickness of the adsorbent layer (Table II).

TABLE II

THE INFLUENCE OF THE THICKNESS OF THE ALUMINA LAYER ON THE R_F VALUES OF SOME 17-KETOSTEROIDS

Steroids	R_F	
	Thickness of Al_2O_3-layer	
	0.9 mm	*2.7 mm*
Androstane-3,17-dione	0.98	0.90
5-Androsten-3β-ol-17-one	0.58	0.46
Androstan-3α-ol-17-one	0.53	0.43
Androstan-3α-ol-11,17-dione	0.23	0.17
Etiocholan-3α-ol-11,17-dione	0.14	0.12
Etiocholane-3α,11β-diol-17-one	0.06	0.05

Repeated development using different solvents

For the repeated development using different solvents with different distances for the respective solvent fronts, PRUSÍKOVÁ[3] derived a general equation, which slightly modified[4] is as follows:

$$"R_F" = a/b \cdot R_1 (1 - R_2) + R_2 ,$$

TABLE III

THE R_F VALUES OF SOME 17-KETOSTEROIDS AFTER REPEATED DEVELOPMENT WITH DIFFERENT SOLVENTS FOR THE SAME DISTANCE

	Mobile phase					
	Chloroform	*Diethyl ether*	*1. Chloroform 2. Diethyl ether*		*1. Diethyl ether 2. Chloroform*	
	R_1	R_2	"R_F"		"R_F"	
			Found	*Calculated*	*Found*	*Calculated*
5-Androstene-3β-ol-17-one	0.49	0.84	0.86	0.92	0.97	0.92
Androstane-3α-ol-11,17-dione	0.38	0.39	0.60	0.62	0.49	0.62
Etiocholane-3α-ol-11,17-dione	0.33	0.22	0.45	0.48	0.36	0.48
Etiocholane-3α,11β-diol-17-one	0.21	0.32	0.48	0.46	0.29	0.46

where "R_F" is the resulting location of the spot with respect to the second front, R_1 and R_2 are the R_F values in the first and second system, respectively, and a and b are the distances of the front after the first and second development, respectively. This relationship was shown to be valid for the separation of steroids using spread-layer chromatography by the agreement between the experimental results and the theoretical calculation according to the above equation, providing that the first solvent did not substantially alter the activity of the adsorbent, *i.e.*, by the use of less polar solvents. The addition of alcohols, the more polar ethers, or esters to the mobile phase during the first development has a definite influence on the results. The use of two different solvents is justified especially if the solvents differ in their behaviour; sometimes it is advantageous to use a combination of two solvents having electron donor and electron acceptor properties, respectively. Some results are shown in Table III.

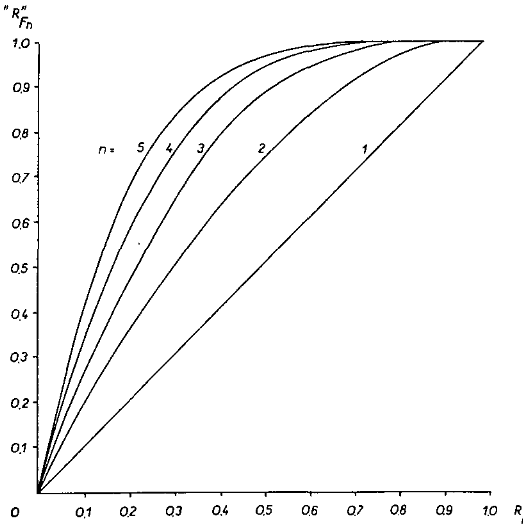

Fig. 1. The apparent "R_F" value after the n-th development in the same system to the identical front distance for any original R_F.

Multiple repeated development in the same system

Multiple repeated development with only one solvent system is now widely employed and has proved successful in many practical cases. If the chromatogram is developed with the same solvent always to the same distance, the equation for the second development is simplified to:

$$"R_F"_2 = 2R - R^2$$

and, for the n-th development it is:

$$"R_F"_n = R\,(1 - R_{n-1}) + R_{n-1},$$

where $"R_F"_n$ is the apparent R_F after the n-th development, R is the R_F value after the first and R_{n-1} the value after the $(n-1)$-th development. The graphic representation of the resulting mobility related always to the constant solvent front—$"R_F"$—for the first to fifth development and any arbitrary original R_F is given in Fig. 1.

TABLE IV

THE R_F VALUES AFTER THE n-th TIME OF REPEATED DEVELOPMENT WITH THE SAME SOLVENT FOR THE SAME DISTANCE

	$n=$	1	2	3	4
Mobile phase: benzene–ethanol (98:2)					
5-Androsten-3β-ol-17-one	found	0.14	0.24	0.32	0.40
	calc.	—	0.26	0.36	0.45
4-Androstene-3,17-dione	found	0.33	0.50	0.62	0.73
	calc.	—	0.55	0.70	0.79
Mobile phase: tetrachloromethane					
Sudan red	found	0.15	0.30	0.42	0.51
	calc.	—	0.28	0.41	0.50
Sudan yellow	found	0.32	0.53	0.68	0.80
	calc.	—	0.54	0.68	0.78
Azobenzene	found	0.66	0.90	0.95	front
	calc.	—	0.88	0.97	0.99

The validity of this relationship was verified by a repeated development of several 17-ketosteroids in benzene–ethanol (98:2) and in tetrachloromethane and is shown in Table IV. It is also valid in those cases where the activity of alumina may be slightly affected by the mobile phase employed.

SUMMARY

The relationship for $"R_F"$ of some steroids after repeated development in different systems was found to be valid in spread-layer chromatography and a relationship for the mobility of some steroids developed several times in the same system was also established.

REFERENCES

[1] R. Neher and A. Wettstein, *Helv. Chim. Acta*, 35 (1952) 276.
[2] J. Barrolier and J. Heilmann, *Z. Physiol. Chem.*, 309 (1957) 221.
[3] M. Prusíková, *Experientia*, 15 (1959) 460.
[4] L. Stárka, *J. Chromatog.*, 4 (1960) 334.
[5] P. Lisboa, *Acta Endocrinol.*, in the press.
[6] M. Mottier and M. Potterat, *Anal. Chim. Acta*, 13 (1955) 46.
[7] V. Černý, J. Joska and L. Lábler, *Collection Czech. Chem. Commun.*, 26 (1961) 1658.
[8] L. Stárka and J. Malíková., *J. Endocrinol.*, 22 (1961) 215.
[9] S. Heřmánek, V. Schwarz and Z. Čekan, *Collection Czech. Chem. Commun.*, 26 (1961) 1669.
[10] V. Schwarz and K. Syhora, *Collection Czech. Chem. Commun.*, 28 (1963) 101.
[11] O. Adamec, J. Matis and M. Galvánek, *Lancet*, I (1961) 81.

CHROMATOGRAPHIC ANALYSIS OF BENZOQUINONES

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Chromatography is well recognized today as an indispensable technique in the study of all kinds of natural pigments. A large proportion of these pigments, occurring in animals, plants and micro-organisms, consists of quinones. Many systems for separating anthraquinones[1–5] and naphthaquinones[2,6,7] have been described, but except for a number of investigations associated with specific biochemical problems (*e.g.* the ubiquinones[8–10]) little work has been reported on the chromatography of benzoquinones in general[11,12]. In connection with analytical work on mould products, a chromatographic method was required that would give a precise and good separation of mixtures containing isomeric *p*-benzoquinones in different stages of hydroxylation. Thin-layer chromatography was chosen as the principal technique as it was rapid and required a smaller amount of the substance to be analysed, but for special purposes a paper chromatographic method was also required. It often proved advantageous to purify the mould extracts on paper chromatograms, and then, after elution, to identify the different quinones on thin-layer plates.

The compounds investigated could be divided into two groups, which had to be analysed separately:

(1) Benzoquinone derivatives where the substituents consisted of one or more methyl, ethyl or methoxyl groups.

(2) Benzoquinone derivatives where in addition at least one of the substituents was a hydroxyl group.

For quantitative determination of the compounds, the ultraviolet absorption curves of the pure products were determined in anhydrous ether, using 1 cm cells. Maximum absorption is found between 265 and 280 mμ[13], and a wavelength of 270 mμ was chosen to obtain standard curves.

MATERIALS AND METHODS

Samples

The quinones used in the present investigation were synthesized in our laboratory, or obtained through the courtesy of other laboratories. The purity was checked by chromatography. Six of the compounds have not been described previously:

The three isomeric ethyldimethylhydroxybenzoquinones (Nos. 12–14 in Table II) were obtained in good yield from the corresponding hydroquinones[14] by oxidation with air in 0.1 *M* phosphate buffer (pH 8.0). After acidification of the buffer solution with concentrated hydrochloric acid the quinones were extracted with ether and purified by sublimation.

Treatment of the ethyldimethylhydroxybenzoquinones with diazomethane in ether solution and subsequent removal of ether gave a quantitative yield of the corresponding methoxy derivatives (Nos. 9–11 in Table III) in the pure form. Data of these six quinones are given in Table I.

TABLE I

DATA OF SIX SYNTHESIZED BENZOQUINONES

Compound	*m.p.*	*Colour*	
		Crystals	*Alkaline solution*
2-Hydroxy-3,6-dimethyl-5-ethyl-1,4-benzoquinone	106°	yellow	violet
2-Hydroxy-3,5-dimethyl-6-ethyl-1,4-benzoquinone	69°	orange	violet
2-Hydroxy-5,6-dimethyl-3-ethyl-1,4-benzoquinone	79°	yellow	violet
2-Methoxy-3,6-dimethyl-5-ethyl-1,4-benzoquinone	135°	yellow	yellow
2-Methoxy-3,5-dimethyl-6-ethyl-1,4-benzoquinone	82°	yellow	yellow
2-Methoxy-5,6-dimethyl-3-ethyl-1,4-benzoquinone	40°	yellow	yellow

Paper chromatography

The compounds were introduced onto Whatman No. 1 paper by the usual spotting technique, and the papers developed by the descending method for a period of 16–24 h, depending on the solvent system used. During this time the solvent descended to 40 cm from the base line. Greatly increased separation of compounds with relatively low R_F values was obtained by allowing the solvent front to overrun the edge of the paper. In these cases, R_F values were determined by simultaneously running spinulosin (No. 30 in Table III) as a reference substance.

Paper chromatographic purification

The mould extracts were introduced as bands along the base line and the chromatograms developed as above. In the solvent system used, all the compounds of Group I moved with or near the solvent front, and could thereby easily be separated from the members of Group II by elution with ethanol. The quinones of Group II could often be identified directly on the paper chromatogram, but if necessary these were also cut out from the paper, eluted with ethanol and identified by thin-layer chromatography.

Thin-layer chromatography

Smooth glass plates (15 × 15 × 0.3 cm) were covered with a thin, even layer of Silica Gel G (Merck, Darmstadt) by spreading a well-stirred mixture of 30 g Silica Gel G and 60 ml of distilled water with a thin-layer applicator. The plates were activated by heating for 1 h at 105°, and placed in a desiccator over calcium chloride. Five microliter drops containing 1–10 μg of the quinones dissolved in ether or ethanol were applied at a distance of 1.5 cm from the edge of the plate. The origin and a front 10 cm above it were marked off, and ascending chromatograms run. When the solvent front reached the 10 cm mark (20–40 min) the plates were removed and airdried.

Quantitative analysis

After 1 min. exposure to gaseous hydrochloric acid the spots were removed by

scraping the silica gel from the plates and transferred quantitatively to a small column, from which the compounds were eluted, using ether, a total volume of 3 ml being collected. The ultraviolet absorption of each compound at 270 mμ was then determined, and the values obtained interpolated in the standard curves. The results were easily reproducible, giving a recovery of 95–100 % of the compound

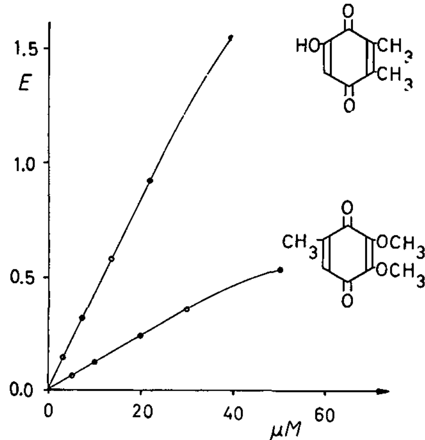

Fig. 1. Extinction at 270 mμ (1 cm cells) as function of concentration for two representative benzoquinones in ethereal solution.

chromatographed. In concentrations lower than 20 μM the quinones obey Lambert-Beer's law, with molecular extinction coefficients ranging from $5 \cdot 10^3$ to $5 \cdot 10^4$ M^{-1}cm^{-1}. Fig. 1 shows the standard curves for one representative compound of each group.

Detection

The chromatographic spots are self-indicating. The quinones of Group I all give yellow spots, those of Group II give colours that are characteristic for their structure, passing from orange to purple or blue with increasing number of substituents, and in some cases enable overlapping spots to be identified. The lower limit of detectability is 1 μg for Group I and 0.1 μg Group II. Not more than 20 μg should be applied to the thin-layer plates.

Solvent systems

The following solvent systems proved satisfactory:

A. Chloroform
B. Chloroform–benzene (3:1 by vol.)
C. Chloroform–xylene (3:1 by vol.)
D. Ethanol–concentrated ammonia (5:1 by vol.)
E. Ethanol–*n*-butanol–2 *M* ammonium hydroxide (3:5:3 by vol.)
F. Propanol–*n*-butanol–2*M* ammonium hydroxide (6:1:3 by vol.).

The first three solvents were used for thin-layer analysis of Group I, the others for Group II. Solvent F was also used for paper chromatography.

RESULTS AND DISCUSSION

Tables II and III list the R_F values of Groups I and II respectively of the investigated quinones. These figures are an average of three or more runs, and the reproducibility was established by a statistical analysis of 20 experiments in which the mobility of three compounds of each group was determined in the different solvent systems. On thin-layer plates, using solvents B-F, the standard deviation of the R_F values was in the order of 5 % of the means for all the compounds. With solvent A, and on paper chromatograms (solvent F) the standard deviation was slightly higher (10 %). The major source of error is attributable to temperature fluctuations with their subsequent effect on the mobilities.

The order in which the quinones of Group I appear on the chromatograms is not much affected by the solvent system used. Many systems have been investigated, and the three given above, based on chloroform, showed the best differentiation of the R_F values. Sometimes two-dimensional chromatography is advantageous, and Fig. 2 shows such a chromatogram, where solvent A has been used for the first dimension and B for the second one.

In most of the 90 or so solvents tested the compounds of Group II showed extensive tailing, which could be avoided only by using alkaline systems, the best systems being based on alcohols containing ammonia. The water-free solvent D gives good resolution and generally very distinct, flattened spots. A difference of 0.02 in R_F value is enough to yield two entirely separated spots if not too much substance

TABLE II

100 × R_F VALUES OF 21 DERIVATIVES OF 1,4-BENZOQUINONE BELONGING TO GROUP I

No.	*Substituents in position*				*Solvent system used*							Colour
					Thin-layer						*Paper*	
	2	*3*	*5*	*6*	*A**	*B**	*C**	*D*	*E*	*F*	*F*	
1	H	H	H	H	66	48	50	73	84	75	85	yellow
2	CH_3	H	H	H	74	53	54	88	91	85	95	yellow
3	CH_3	H	H	CH_3	77	47	52	84	92	81	96	yellow
4	CH_3O	H	H	H	50	54	35	90	92	90	92	yellow
5	CH_3	CH_3O	H	H	54	36	40	87	90	85	93	yellow
6	CH_3	H	CH_3O	H	33	31	26	80	78	80	95	yellow
7	CH_3	H	H	CH_3O	52	22	33	85	87	73	100	yellow
8	CH_3	CH_3	CH_3O	H	43	42	40	76	85	81	100	yellow
9	CH_3	CH_3O	CH_3	H	61	55	67	85	88	85	97	yellow
10	CH_3	CH_3O	H	CH_3	24	21	20	81	78	88	96	yellow
11	CH_3	CH_3	CH_3	CH_3O	63	58	62	90	91	95	100	yellow
12	CH_3	CH_3	C_2H_5	CH_3O	90	66	64	95	96	92	100	yellow
13	CH_3	C_2H_5	CH_3	CH_3O	84	67	62	95	90	91	100	yellow
14	CH_3	C_2H_5	CH_3O	CH_3	87	63	64	92	94	90	100	yellow
15	CH_3O	H	CH_3O	H	15	16	10	83	83	88	92	yellow
16	CH_3O	H	H	CH_3O	17	19	15	75	81	80	95	yellow
17	CH_3	CH_3O	CH_3O	H	39	26	23	78	84	77	100	yellow
18	CH_3	CH_3O	H	CH_3O	36	29	26	83	80	73	98	yellow
19	CH_3	H	CH_3O	CH_3O	46	32	30	79	85	73	95	yellow
20	CH_3	CH_3O	CH_3O	CH_3	43	36	30	82	88	79	100	yellow
21	CH_3	CH_3O	CH_3O	CH_3O	48	39	27	85	89	75	98	yellow

* Solvent systems suitable for separations within the group.

TABLE III

100 × R_F VALUES OF 31 DERIVATIVES OF 1,4-BENZOQUINONE BELONGING TO GROUP II

No.	*Substituents in position* 2	3	5	6	*Solvent system used* *Thin-layer* *A*	*B*	*C*	*D**	*E**	*F**	*Paper* *F**	*Colour*
1	OH	H	H	H	6	4	4	74	78	79	38	orange
2	CH_3	OH	H	H	2	1	1	64	70	68	78	orange
3	CH_3	H	OH	H	0	0	0	73	67	63	70	red-orange
4	CH_3	H	H	OH	0	0	0	44	58	52	dec.	orange
5	CH_3	CH_3	OH	H	11	5	3	65	70	67	71	orange
6	CH_3	OH	CH_3	H	11	5	4	76	64	64	79	violet
7	CH_3	OH	H	CH_3	22	8	10	74	62	68	77	red-violet
8	CH_3	CH_3	CH_3	OH	22	9	9	58	63	58	79	red-violet
9	CH_3	CH_3	C_2H_5	OH	29	14	30	66	67	68	77	violet
10	CH_3	C_2H_5	CH_3	OH	23	10	24	65	64	60	77	violet
11	CH_3	C_2H_5	OH	CH_3	17	7	11	72	68	64	80	violet
12	OH	H	OH	H	0	0	0	0	0	0	4	blue-violet
13	OH	H	H	OH	0	0	0	21	25	25	13	orange
14	OH	H	CH_3O	H	5	2	1	50	60	54	56	red-violet
15	OH	H	H	CH_3O	0	0	0	62	62	67	52	orange
16	CH_3	OH	OH	H	0	0	0	42	16	29	32	violet
17	CH_3	OH	H	OH	0	0	0	6	20	10	19	red-violet
18	CH_3	H	OH	OH	1	1	1	63	63	60	65	violet
19	CH_3	OH	CH_3O	H	8	2	5	66	63	67	60	violet
20	CH_3	CH_3O	OH	H	4	2	3	50	38	45	55	orange
21	CH_3	OH	H	CH_3O	0	0	0	64	59	62	54	blue
22	CH_3	CH_3O	H	OH	0	0	0	60	58	58	51	violet
23	CH_3	H	OH	CH_3O	0	0	0	26	38	30	30	violet
24	CH_3	H	CH_3O	OH	1	1	1	49	56	49	45	violet
25	CH_3	CH_3	OH	OH	0	0	0	45	41	41	53	violet
26	CH_3	OH	CH_3	OH	0	0	0	21	15	18	23	red-violet
27	CH_3	OH	OH	CH_3	7	3	4	54	64	63	38	blue
28	CH_3O	CH_3O	OH	H	11	5	6	67	65	55	70	blue-violet
29	CH_3	OH	OH	OH	0	0	0	0	0	0	9	blue
30	CH_3	OH	CH_3O	OH	0	0	0	5	0	0	25	blue-violet
31	CH_3	OH	CH_3O	CH_3O	3	1	1	65	65	58	69	blue-violet

* Solvent systems suitable for separations within the group.

is applied to the plates. There is, however, still some tailing of the quinones with two hydroxyl groups. With solvent E all compounds develop well-defined spots, slightly more diffuse than obtained with D. The resolution is good and the composition of the system can be varied to meet special needs for separation. Increasing the percentage of butanol makes the system more adapted to the chromatography of the less polar quinones, but also increases the time of development. Decreasing the percentage of butanol gives better separation of compounds with low R_F values. Butanol should not be completely omitted, as the spots then tend to tail too much. Variation of the proportion of $2M$ ammonium hydroxide is of no use, as it is chosen to be optimum as regards avoidance of tailing and resolution ability. Solvent F has the same general properties as solvent E.

Paper chromatography was used partly to separate Group I from Group II, partly to identify the members of Group II. For these purposes, solvent F proved superior to other systems, and was exclusively used. It appears from Table III that

paper chromatography gives as good separations within Group II as the thin-layer technique, particularly between the more polar compounds. A better differentiation of the less polar quinones can be effected by increasing the proportion of butanol. The long time of development of the paper chromatograms is, however, a great disadvantage as some decomposition of the less stable quinones (*e.g.* 3-hydroxy-2,5-

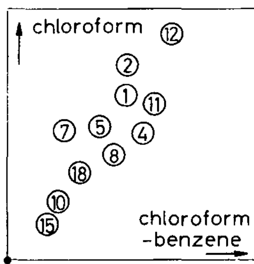

Fig. 2. Two-dimensional chromatography of benzoquinones from Group I. The numbers of the compounds refer to Table II.

toluquinone) occurs in the alkaline medium. Consequently, quantitative analysis should be performed only on thin-layer plates.

No general straight-line relationship between R_F values and number of substituents could be obtained, but in most cases the R_F values are increased by methyl groups, decreased by methoxyl groups, and decreased still more by hydroxyl groups.

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SUMMARY

A satisfactory method for the separation, identification and determination of *p*-benzoquinone derivatives by adsorption chromatography on plates and partition chromatography on paper, using different solvents as developer, is described, and the dependence of the R_F values on the nature of the solvent system discussed.

REFERENCES

1 J. FRANC, *Chem. Listy*, 49 (1955) 872.
2 J. GASPARIČ, *Mikrochim. Acta*, (1958) 681.
3 J. FRANC, *Collection Czech. Chem. Commun.*, 24 (1959) 250.
4 J. FRANC AND M. WURST, *Collection Czech. Chem. Commun.*, 25 (1960) 657.
5 J. GASPARIČ, *J. Chromatog.*, 4 (1960) 75.
6 L. REIO, *J. Chromatog.*, 1 (1958) 338.
7 M. BARBIER AND E. LEDERER, *J. Chromatog., Chromatog. Data*, 1 (1958) xlii.
8 A. DIPLOCK, J. GREEN, E. EDWIN AND J. BUNYAN, *Biochem. J.*, 76 (1960) 563.
9 H. WAGNER, L. HÖRHAMMER AND B. DENGLER, *J. Chromatog.*, 7 (1962) 211.
10 J. GREEN, S. MARCINKIEWICZ AND D. MCHALE, *J. Chromatog.*, 10 (1963) 158.
11 M. BARBIER, *J. Chromatog.*, 2 (1959) 649.
12 H. SCHILDKNECHT, *Angew. Chem.*, 69 (1957) 62.
13 W. FLAIG, J. C. SALFELD AND E. BAUME, *Ann.*, 618 (1958) 117.
14 I. LJUNGCRANTZ AND K. MOSBACH, *Acta Chem. Scand.*, in the press.

THE OCCURRENCE OF AMINES IN HUMAN URINE: DETERMINATION BY COMBINED ION EXCHANGE AND PAPER CHROMATOGRAPHY*

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Certain biogenic amines, such as epinephrine, norepinephrine, serotonin, and histamine, have been known for some time to be of physiological importance. It is reasonable to suppose that some among the many other amines that are excreted in human urine may also have important functions in some tissues of the body.

Biogenic amines are likely candidates for the role of synaptic transmitters within the central nervous system, and the differential distribution of such amines as serotonin, norepinephrine, and dopamine within the brain suggests their possible relationship to specialized functions. Other chemically related amines, such as bufotenin, N,N-dimethyltryptamine, psilocin, and mescaline, are known to have psychotomimetic effects in man. Pharmacological modification of the metabolism of such amines may be associated with altered brain function. For example, a recent study[1] suggests the possible implication of toxic amines in the etiology of schizophrenia, since an exacerbation of mental dysfunction occurred in certain schizophrenic patients on monoamine oxidase blockade after the administration of oral loads of tryptophan or methionine. Thus, a knowledge of the normal pattern of urinary excretion of amines in man, and an identification of the unknown amines regularly present in urine, should be useful not only as a basis for the study of a wide variety of metabolic diseases, but in particular for exploration of the mechanisms of mental dysfunction.

With this view in mind, a preliminary study of the urinary excretion of amines in normal children was carried out. Although more than 40 amines were found to be regularly present in urine, less than half of these could be identified[2]. In the course of the investigation, it became obvious that two-dimensional paper chromatography alone was not sufficient for a complete separation and identification of these amines, even after they had been concentrated and separated from amino acids and the acidic and neutral constituents of urine.

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Several investigators have developed ion exchange chromatographic techniques for purifying individual amines originally present in physiological fluids[3-5]. The experiments reported here were designed to devise a method of separating the components of a complex mixture of amines by the consecutive use of ion exchange column chromatography and paper chromatography and to apply the method to a study of the amines present in the urine of children. The results have confirmed the tentative identification[2] of several amines not previously reported in human urine and have shown that the number of amines regularly present in urine is greater than had been demonstrated previously through the use of paper chromatography alone. The method provides a means for the isolation of unidentified urinary amines in relatively pure form.

MATERIAL AND METHODS

General

Preliminary experiments with authentic amines demonstrated the impracticability of separating both aliphatic and aromatic amines on a single ion exchange resin or with a single developing buffer. Although aliphatic monoamines could be separated on columns of Amberlite CG-120, aliphatic diamines and aromatic amines were so firmly bound to the resin that they could be eluted only by strongly basic buffers, which either destroyed them or made subsequent paper chromatography difficult. The aromatic amines and aliphatic diamines could be separated on columns of Amberlite CG-50, but on this resin many aliphatic monoamines were so readily eluted that little or no separation occurred. Therefore, a procedure was adopted in which both ion exchange resins were employed.

Sources of amines

Most of the authentic amines used in this investigation were obtained from commercial sources. *o*-Tyramine was prepared enzymatically from *o*-tyrosine by incubation with *Streptococcus fecalis* decarboxylase, and *p*-hydroxybenzylamine was prepared from *p*-methoxybenzylamine by demethylation with hydriodic acid. Five amines were obtained as gifts from other investigators*.

Preparation of developers

Because the amine fractions from the ion exchange columns were later to be chromatographed on paper, it was desirable to use volatile developers for the column chromatograms. Four aqueous pyridine–acetic acid buffers were prepared. Their composition and the quantities of reagents used to prepare them are shown in Table I.

Reagent grade glacial acetic acid and redistilled reagent grade pyridine were used. The normality of the pH 6.32 and pH 6.12 buffers refers to their acetic acid content, while the normality of the pH 5.50 and pH 3.50 buffers refers to their pyridine content.

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TABLE I

COMPOSITION OF AQUEOUS VOLATILE BUFFERS (PER LITER)

pH	3.50	5.50	6.12	6.32
Normality	0.2 N*	0.8 N*	0.2 N**	0.1 N**
Reagents (ml)				
Pyridine	16.1	64.4	175	150
Glacial acetic acid	115	21.0	11.5	5.75

* Normality in terms of pyridine.
** Normality in terms of acetic acid.

Chromatography of aromatic monoamines and aliphatic diamines

Amberlite CG-50, type 2, a weakly acidic carboxylic acid type cation exchange resin, was first purified initially according to the procedure described by HIRS *et al.*[6]. A 100-g portion of resin in the hydrogen form was suspended in 300 ml of pH 6.32 buffer and was stirred for 20 min. Sufficient pyridine was added to raise the pH of the suspension to pH 6.32 ($\pm$ 0.05). After the resin had been filtered off on a coarse sintered glass Büchner funnel and washed 3 times with 100 ml of pH 6.32 buffer, it was suspended in 2 volumes of this buffer and stored at 5° until used.

A 45 cm column of Amberlite CG-50 was poured in sections in a jacketed glass tube with an internal diameter of 0.9 to 1.0 cm. The suspension of resin was preheated to 40° before pouring the column, which was maintained constantly at 40° throughout pouring, equilibration, and the running of the chromatogram in order to prevent bubble formation and shrinkage. The column was equilibrated with at least 100 ml of pH 6.32 buffer before commencing the chromatogram. Passage of developer during equilibration and during the subsequent chromatogram was controlled with a Milton Roy Chromatographic Minipump*.

The sample containing from 0.02 to 0.1 mmole of authentic amines was dissolved in 2 ml of pH 6.32 buffer, applied directly to the top of the resin column, and rinsed in with small portions of developer. The chromatogram was first developed with pH 6.32 buffer at a flow rate of 10 ml/h, and 2-ml fractions were collected. After 25 h, or when 250 ml of effluent had been collected, the developing solution above the resin column, in the pump, and in the reservoir was replaced with pH 6.12 buffer. The chromatogram was then continued at the same flow rate for another 25 h, or until a total of 500 ml of effluent had been collected.

Chromatography of aliphatic monoamines

Amberlite CG-120, a strongly acidic sulfonic acid type cation exchange resin, was initially prepared by the hydraulic separation method of MOORE *et al.*[7]. A 100-ml portion of settled resin from Fraction C was washed on a coarse sintered glass Büchner funnel successively with 600 ml of N sodium hydroxide, 600 ml of water, 600 ml of 2 N hydrochloric acid, 600 ml of water, 400 ml of N pyridine, and finally with three 200-ml portions of pH 3.50 buffer. The resin was then suspended in 2 volumes of this buffer and was stored at 5° until used.

A 30 cm column of Amberlite CG-120 was poured in sections in a jacketed glass tube with an internal diameter of 0.9 to 1.0 cm. Packing of the column was done at

* Milton Roy Co., Philadelphia, Pa.

room temperature and facilitated by the use of air pressure at 30 cm of mercury. The resin column was equilibrated at 50° with at least 100 ml of pH 3.50 buffer. The pump was used with this type of chromatogram also.

The sample containing from 0.02 to 0.1 mmole of authentic amines was dissolved in 2 ml of pH 3.50 buffer, the pH was then reduced to approximately 2.2 by the addition of 0.06 ml of 6 *N* hydrochloric acid, and the sample was applied directly to the top of the resin column and rinsed in with small portions of developer. The chromatogram was first developed with pH 3.50 buffer at a flow rate of 30 ml/h and a temperature of 50°; 2-ml fractions were collected. After 600 ml of effluent had been collected, the developing solution above the resin column, in the pump, and in the reservoir was replaced with pH 5.50 buffer. The chromatogram was then continued with this second developer at the same temperature and flow rate for a further 250 ml.

The above conditions do not elute diamines from the chromatogram. It was possible, however, to detect diamines if, after the chromatograms were completed, the columns were eluted with 2 *N* sodium hydroxide. These strongly basic eluates were then neutralized with acetic acid, and after evaporation to dryness, the diamines were extracted with ethanol and acetone.

Examination of effluent fractions

Each fraction of effluent was then examined by reaction with ninhydrin on the Technicon Auto Analyzer* by means of the procedure of SCHROEDER *et al.*[8]. Color produced by ninhydrin-reactive amines was scanned at 570 mμ. When authentic amines which yielded a yellow color with ninhydrin were chromatographed, optical density was also recorded at 440 mμ.

Many of the authentic amines that were chromatographed produce no color with ninhydrin and cannot be located in the effluent in this way. Some, indeed, emerge in mixture with ninhydrin-positive substances. The ninhydrin-negative amines had to be detected by other means. After all fractions had been examined for reaction with ninhydrin as described above, those fractions that contained discrete peaks of ninhydrin-positive material were pooled separately. Of those fractions that contained no ninhydrin-positive material, every three to five were arbitrarily pooled.

Pooled fractions of effluent were then concentrated to dryness by removing the volatile buffer under reduced pressure on a rotary evaporator. The residual material was dissolved in a small volume of methanol and subjected to one- or two-dimensional chromatography on paper. The amines were detected by spraying the paper chromatograms with appropriate reagents for ninhydrin-negative as well as ninhydrin-positive amines. The solvents and spray reagents for the paper chromatography of amines have been described in detail elsewhere[2].

Subjects and patients

Urine specimens were obtained from 7 normal children and from 2 juvenile psychotics. The free and conjugated amines were separated from other urinary constituents and were concentrated approximately 1000-fold by a technique previously described[2]. Aliquot portions of these concentrates equivalent to volumes of original urine that contained 250 to 350 mg of creatinine were chromatographed on ion exchange columns of the same size in the same manner as has been described above.

* Technicon Instruments Corporation, Chauncey, N. Y.

TABLE II

ELUTION VOLUMES OF AMINES CHROMATOGRAPHED ON AMBERLITE CG-50*

Compound	*Range of elution (ml)*	*Elution peak** (ml)*	*Compound*	*Range of elution (ml)*	*Elution peak** (ml)*
Histidine	25–31	28	3-Hydroxy-4-methoxyphenylethylamine	145–163	
1-Methylhistidine	25–31	28	Putrescine	140–171	159
Arginine	31–42	35	*p*-Hydroxybenzylamine	151–171	162
Ethanolamine	32–40	36	Cadaverine	163–183	
Ammonia	33–42	38	Dopamine	163–190	
Ethylamine	38–46	42	Benzylamine	169–189	178
Pyrrolidine	49–58	54	*p*-Tyramine	170–196	186
N-Acetylhistamine	49–65		3-Ethoxy-4-hydroxybenzylamine	177–193	
Pyridoxamine	68–79	74	*m*-Tyramine	183–202	
N-Methylmetanephrine	78–89		*p*-Methoxybenzylamine	192–215	203
Methanephrine	102–115	109	Histamine	202–225	214
Epinephrine	101–124		Bufotenin	205–235	
Normetanephrine	112–130	121	Phenylethylamine	228–254	240
1-Methylhistamine	112–130	121	*o*-Tyramine	218–262	241
Norepinephrine	116–132		*p*-Methoxyphenylethylamine	228–261	244
Synephrine	118–135		Kynuramine	229–261	244
Isoamylamine	119–136	128	2,2′-Dithiobis-(ethylamine)	277–297	289
Mescaline	124–142		Serotonin	283–303	
3,4-Dimethoxybenzylamine	127–142		Agmatine	305–321	312
Octopamine	132–147		N,N-Dimethyltryptamine	310–335	
3-Methoxy-4-hydroxybenzylamine	134–150		5-Methoxytryptamine	328–352	339
Epinine	135–151		Tryptamine	380–415	397
3,4-Dimethoxyphenylethylamine	140–154		Spermidine	408–466	438
3-Methoxytyramine	141–158		5-Methyltryptamine	465–505	483

* Authentic compounds were chromatographed in mixtures on Amberlite CG-50 columns 45 cm in length and 0.9 to 1.0 cm in diameter, at a flow rate of 10 ml/h and a temperature of 40°. Chromatograms were developed with pH 6.32—0.1 *N* pyridine *acetate* buffer for the first 250 ml, and thereafter with pH 6.12—0.2 *N* pyridin *acetate* buffer.

** Elution peaks were not obtained for a number of amines giving no color or weak colors with ninhydrin.

The aliphatic monoamines in the urines of 4 normal children and 1 psychotic child were separated on Amberlite CG-120 columns. One of the normal children had been maintained on a plant-free diet and had been given neomycin orally for 5 days prior to urine collection in order to eliminate amines of exogenous plant origin from the urine and to minimize those formed by the bacterial flora of the intestinal tract.

Aromatic amines and aliphatic diamines in the urines of 3 normal children and one psychotic child were separated on Amberlite CG-50 columns. Two of the normal children so studied were administered monoamine oxidase inhibitors (nialamide or pheniprazine) prior to and during urine collections.

RESULTS

Chromatography of authentic amines

The elution volumes of a number of authentic aromatic or heterocyclic amines and of several aliphatic polyamines from Amberlite CG-50 are listed in Table II. Also shown are the emergence points of several basic amino acids and aliphatic monoamines commonly present in urinary amine concentrates. These compounds are always eluted from Amberlite CG-50 at the same effluent volumes when the columns are operated under the conditions described.

The following observations were made concerning the effect of chemical structure on the elution volume from Amberlite CG-50. Methylation of the amino group hastens elution from the resin, and tertiary amines are eluted more rapidly than secondary amines. Acetylation of the amino group accelerates elution very markedly. Hydroxylation of the β carbon atom of the side chain of phenylethylamine derivatives speeds elution from the resin. Hydroxylation of the benzene ring of aromatic amines hastens their emergence from the resin, but this effect is greatest when the ring is hydroxylated in the *para* position and least in the *ortho* position. Methylation of a single hydroxyl group in the ring of an aromatic amine generally causes the emergence point of the compound to revert to that of the original unhydroxylated analogue. If two or more ring hydroxyl groups are methylated, however, the opposite holds true, and the compound is eluted more rapidly. The longer the carbon chain of aliphatic amines or the side chain of aromatic amines, the more elution from Amberlite CG-50 is delayed. The presence of the indole nucleus slows elution. Increase in the number of amino groups in polyamines delays elution, and when as many as four are present, as in spermine, elution from the resin cannot be effected with the developing buffers used.

Table III presents the elution volumes of a group of authentic aliphatic monoamines from Amberlite CG-120 columns that were operated as described above. Included are the elution volumes of ammonia and the basic amino acids to be found in urinary amine concentrates.

The following observations were made concerning the effect of varying chemical structure on the elution volumes of aliphatic monoamines on Amberlite CG-120. Substitution of a methyl group on the amino group hastens elution from the resin. Hydroxylation of the aliphatic chain speeds elution, and the greater the number of hydroxyl groups the more rapidly the compound is eluted. Increase in the number of carbon atoms in the aliphatic chain delays emergence from the resin. Whereas diamines fail completely to be eluted from Amberlite CG-120, acetylation of one of the two amino groups results in their early elution.

TABLE III

ELUTION VOLUMES OF AMINES CHROMATOGRAPHED ON AMBERLITE CG-120*

Compound	*Range of elution (ml)*	*Elution peak (ml)*	*Compound*	*Range of elution (ml)*	*Elution peak (ml)*
Glucosamine	115–132	121	γ-Methylmercaptopropylamine sulfoxide	290–300	295
Galactosamine	115–132	121	Ammonia	296–345	308
N-Acetylethylenediamine	145–160	152	*n*-Propylamine	312–332	322
β-Methoxyethylamine	150–165	157	Isobutylamine	316–345	328
N-Methylethanolamine	156–171	163	Hydroxylamine	334–371	347
3-Amino-1-propanol	175–191	183	1-Methylhistidine	340–375	361
Serinol	175–191	183	Cyclopropylamine	350–382	364
Dimethylamine	187–200	193	3-Methylhistidine	353–388	370
β-Hydroxypropylamine	195–209	201	*n*-Butylamine	390–420	405
N-Methylethylamine	198–215	206	Isoamylamine	430–465	445
Diethylamine	212–224	218	γ-Methylmercaptopropylamine	447–490	467
Ethanolamine	210–230	219	*n*-Amylamine	504–542	520
2-Aminobutanol	220–236	228	Histidine	591–629	600
Pyrrolidine	250–270	260	Ornithine	646–656	650
Piperidine	250–272	261	Lysine	653–662	656
Methylamine	262–280	273	Carnosine	657–666	662
Ethylamine	272–294	282	Arginine	820–855	832

* Authentic compounds were chromatographed in mixtures on Amberlite CG-120 columns 30 cm in length and 0.9 to 1.0 cm in diameter, at a flow rate of 30 ml/h and a temperature of 50°. Chromatograms were developed with pH 3.50—0.2 *N pyridine* acetate buffer for the first 600 ml, and thereafter with pH 5.50—0.8 *N pyridine* acetate buffer. The breakthrough of the second developer occurred at 636 ml.

Although the detection of ninhydrin-reactive amines in the effluent fractions is much facilitated by the use of the Technicon Auto Analyzer, the older manual methods for detecting ninhydrin-reactive materials in the effluent of chromatographic columns are equally applicable[9]. Regardless of which ninhydrin procedure is used, it is adequate to determine the absorbance of the reaction mixture at 570 mμ only, because pyrrolidine is the only amine likely to occur in sufficient amount in urine to produce a measurable absorbance at 440 mμ. In our hands, the absolute elution volumes of amines have been very reproducible. In the hands of other investigators, they would no doubt vary somewhat from the figures given in Tables II and III because of differences in the dimensions of the columns, but the relative emergence points of the various amines should not vary.

Ion exchange chromatography as described is not suitable for the detection of small amounts of catecholamines, because they appear to be unstable at the pH of the developing buffer used. Some of the more volatile aliphatic amines, such as methylamine, dimethylamine, ethylamine and piperidine, may partly be lost when effluent fractions are taken to dryness prior to paper chromatography. When γ-methylmercaptopropylamine, the decarboxylation product of methionine, is chromatographed on Amberlite CG-120, part is converted to the sulfoxide. This is not disadvantageous, however, because detection of the sulfoxide in a chromatogram strengthens identification of the parent amine.

Chromatography of amines in urine

Table IV lists the amines regularly present in urine which were detected by ion exchange chromatography on Amberlite CG-50 and subsequent paper chromatography. The unidentified compounds are numbered for the sake of simplicity according to the system of PERRY *et al.*[2], and the solvents and spray reagents used in paper chromatography are those described by these authors. For each compound, Table IV presents its elution volume, its R_F values on paper in four different solvents, and the colors produced by various spray reagents.

Table V lists the amines regularly found in urine amine concentrates and separated on columns of Amberlite CG-120. The numbering of unidentified compounds corresponds to the system previously reported[2]. R_F values in four different solvents are recorded, as well as appropriate color reactions.

In both Tables IV and V, amines have been listed in the order of their emergence from the ion exchange columns. Unidentified compounds have been presumed to be amines because of their chromatographic behavior on resin columns and on paper, as well as their color reactions.

Although the number of urines studied by this technique has been small, the aromatic amines listed are assumed to be present regularly in urine, because they were observed routinely in a much larger group of children's urines that had been studied by paper chromatography alone[2,10]. The aliphatic amines listed in Table V were present in the 5 urines that were chromatographed on Amberlite CG-120. The regular occurrence of some of them, however, is more questionable, because two-dimensional paper chromatographic examination on a larger number of urines had failed to detect their presence. Several additional compounds, presumably amines, that were detected in only one or two of the urines are not listed in Tables IV and V.

(Text continued p. 369)

TABLE IV

URINARY AMINES CHROMATOGRAPHED ON AMBERLITE CG-50*

Compound No.	Identification	Elution zone (ml)	R_F values				Color reactions with spray reagents				
			BuAc	MBF	ANF	IpAm	DPNA	DSA	DQCI	DMCA	Ninhydrin-lutidine
9	Unidentified	46–58	0.44	0.59	0.39	0.46	Orange	Orange-red			
8	N-Acetylhistamine	49–65	0.47	0.71	0.33	0.73	Orange	Orange-red			
42	Unidentified	67–77	0.35	0.43	0.28	0.17					Pink
10	Unidentified	67–83	0.58	0.69	0.63	0.79	Orange	Orange-red			
25	Unidentified	93–103	0.71	0.73	0.70	0.54	Lavender	Orange	Green		
45	Unidentified	99–114	0.52	0.62	0.52	0.50	Pink	Yellow	Blue		
19	Metanephrine	102–115	0.58	0.66	0.57	0.64	Purple	Orange	Blue		Purple (70°)
44	Unidentified	110–120	0.86	0.87	0.92	0.64					Purple
46	Unidentified	113–122	0.63	0.67	0.67	0.61	Blue				
18	Normetanephrine	112–130	0.54	0.56	0.45	0.47	Purple	Orange	Blue		Purple
37	1-Methylhistamine	112–130	0.35**	0.51	0.18	0.57					Purple
15	Synephrine	118–135	0.60	0.65	0.57	0.64	Pink	Yellow	Blue		Purple (70°)
14	Octopamine	132–147	0.57	0.57	0.49	0.50	Pink	Yellow	Blue		Purple
17	3-Methoxy-4-hydroxy-benzylamine	134–150	0.52	0.58	0.62	0.51	Purple	Orange	Blue		Yellow→ yellow-brown
26	Unidentified	139–157	0.63	0.67	0.66	0.65	Purple	Orange	Grey-blue		
24	Unidentified	140–162	0.49	0.57	0.38	0.27	Red-purple	Brown	Red-purple (f.)		

16	3-Methoxytyramine	141–158	0.64	0.64	0.61	0.67	Blue-grey	Orange	Grey		Purple
47	Putrescine	140–171	0.30**	0.32	0.12	0.47					Purple
13	*p*-Hydroxybenzylamine	151–171	0.58	0.62	0.61	0.60	Pink	Yellow	Blue		Yellow→ yellow-brown
48	Cadaverine	163–183	0.30**	0.37	0.13	0.55					Purple
49	Unidentified	173–186	0.31	0.46	0.13	0.76					Purple
11	*p*-Tyramine	170–196	0.62	0.67	0.59	0.72	Lavender	Orange	Grey		Purple
20	Unidentified	174–191	0.53	0.63	0.49	0.58	Yellow	Blue (f.)	Yellow		
12	*m*-Tyramine	183–202	0.67	0.70	0.58	0.69	Purple-pink	Orange-yellow	Blue		Red-purple
7	Histamine	202–225	0.44**	0.27	0.07	0.57	Orange	Orange-red			Purple
3	Bufotenin	205–235	0.61	0.68	0.71	0.86	Red-purple	Brown	Grey	Blue	
4	Kynuramine	229–261	0.66	0.74	0.66	0.78				Purple	Purple
21	Unidentified	230–270	0.75	0.80	0.80	0.85	Pink→ red-purple	Yellow	Blue		
50	2,2′-Dithiobis(ethyl-amine)	277–297	0.30**	0.36	0.15	0.63					Purple
2	Serotonin	283–303	0.51	0.50	0.51	0.63	Red-purple	Brown	Grey	Blue	Yellow-brown
1	Tryptamine	380–415	0.75	0.70	0.72	0.81				Purple	Purple

* Solvents used in paper chromatography were: BuAc = *n*-butanol–acetic acid–water (12:3:5); MBF = 2-methyl-3-butyn-2-ol–formic acid–water (75:5:20); ANF = acetonitrile–formic acid–water (80:2:18); and IpAm = isopropanol–ammonium hydroxide–water (8:1:1). Color reactions are listed for the following reagents[2]: DPNA = diazotized *p*-nitroaniline; DSA = diazotized sulfanilic acid; DQCI = dichloroquinone chloroimide; DMCA = dimethylaminocinnamaldehyde; and ninhydrin–lutidine. Blank spaces indicate failure to give color with a reagent. The symbol (f.) indicates that color fades rapidly. Temperatures shown in parentheses indicate color develops only if sheets are heated.

** Hydrochlorides of these diamines have a lower R_F in BuAc than do the amines after elution from the resin.

TABLE V

URINARY AMINES CHROMATOGRAPHED ON AMBERLITE CG-120*

Compound No.	Indentification	Elution zone (ml)	R_F values				Color with ninhydrin–lutidine	Color after nickel sulfate
			BuAc	MBF	ANF	IpAm		
51	Unidentified	105–118	0.57	0.80	0.79	0.57	Purple (70°)	Pink
52	Unidentified	119–133	0.47	0.66	0.39	0.66	Purple	Pink
53	Unidentified	163–182	0.59	0.73	0.69	(d.)	Grey-purple (100°)	Grey-pink
54	Dimethylamine	187–200	0.48	0.63	0.59	(d.)	Purple (70°)	Pink
32	β-Hydroxypropylamine	195–209	0.48	0.59	0.40	0.65	Purple	Pink
40	Unidentified	195–209	0.41	0.48	0.31	0.53	Yellow	Yellow
30	Ethanolamine	210–230	0.40	0.54	0.28	0.56	Purple	Pink
55	Unidentified	210–230	0.65	0.83	0.75	0.80	Grey (100°)	Pink-grey
56	Unidentified	227–245	0.12	0.38	0.12	0.04	Purple	Pink
57	Unidentified	230–246	0.50	0.61	0.41	0.57	Purple (70°)	Pink
33	Pyrrolidine	250–270	0.53	0.75	0.62	(d.)	Yellow (70°)	Yellow
59	Piperidine	250–272	0.58	0.78	0.65	(d.)	Blue-purple (100°)	Purple
58	Methylamine	262–280	0.41	0.53	0.25	(d.)	Purple	Pink
31	Ethylamine	272–294	0.51	0.65	0.58	(d.)	Purple	Pink
41	Unidentified	247–294	0.31	0.37	(d.)	(d.)	Pink	Pink
44	Unidentified	670–685	0.86	0.87	0.92	0.64	Purple	Grey-pink
42	Unidentified	670–685	0.35	0.42	0.28	0.17	Pink	Salmon

* Solvents used in paper chromatography were: BuAc = *n*-butanol–acetic acid–water (12:3:5); MBF = 2-methyl-3-butyn-2-ol–formic acid–water (75:5:20); ANF = acetonitrile–formic acid–water (80:2:18); and IpAm = isopropanol–ammonium hydroxide–water (8:1:1). The symbol (d.) in the vertical columns listing R_F values indicates the compound is destroyed or volatilizes in that solvent. The final two vertical columns show colors given by compounds when paper chromatograms are sprayed with ninhydrin–lutidine[2] and then countersprayed with nickel sulfate. Temperatures shown in parentheses indicate color develops only if sheets are heated.

The urines of the two psychotic children studied showed all of the compounds listed in Tables IV and V. The identity of an unusual amine detected in the urine of one of these patients and not in that of normal children will be reported elsewhere[11].

Figs. 1 and 2 present graphically the results of chromatograms on Amberlite CG-50 and Amberlite CG-120 respectively of urinary amine concentrates. In these

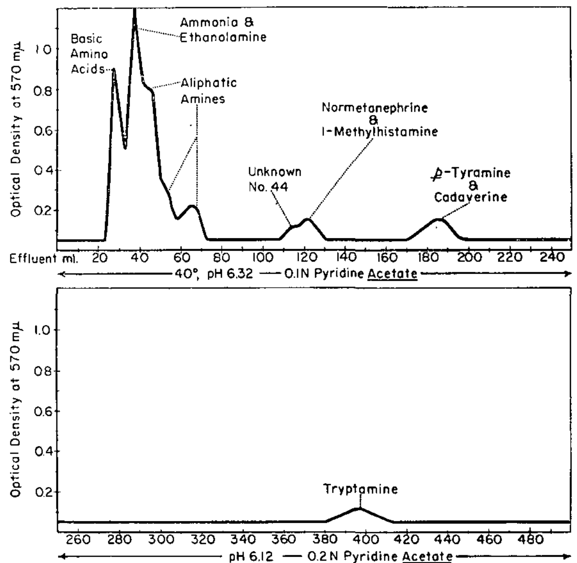

Fig. 1. Chromatographic analysis of ninhydrin-reactive amines and related compounds in normal urine amine extract on a 1 × 45-cm column of Amberlite CG-50.

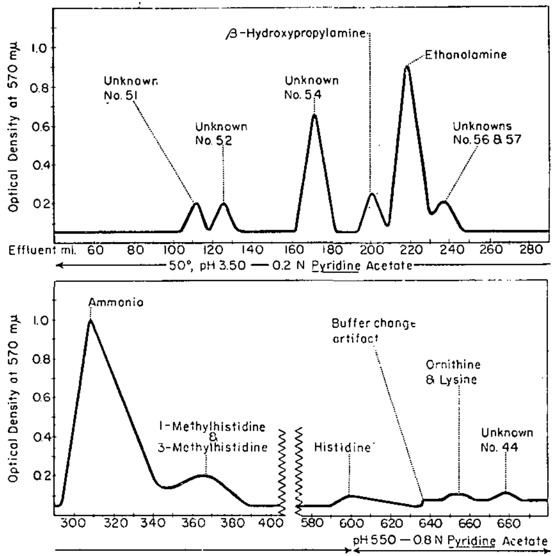

Fig. 2. Chromatographic analysis of ninhydrin-reactive amines and related compounds in normal urine amine extract on a 1 × 30-cm column of Amberlite CG-120.

instances, the urinary amines in 300 mg creatinine equivalents of urine were applied to the resin columns, and the effluent fractions were examined by reaction with ninhydrin on the Technicon Auto Analyzer, recording optical density of the color produced at 570 mμ. The chromatogram depicted in Fig. 1 was that of a child who had been administrated pheniprazine as a monoamine oxidase inhibitor, and the peaks for *p*-tyramine and tryptamine are therefore more prominent than would ordinarily be expected. The wide variety of amines present in urine is not apparent in these graphs, because many aromatic amines, and all the secondary aliphatic amines, fail to produce sufficient color with ninhydrin to register on the recorder. The location of these ninhydrin-negative amines in the column effluent is not difficult to calculate, however, if based upon the emergence points of certain ninhydrin-positive amines, such as those shown for normetanephrine, *p*-tyramine, and tryptamine in Fig. 1.

The present technique has advantages if an amine is present in urine in such low concentration that it cannot easily be detected during routine paper chromatography. By subjecting to paper chromatography the material in the entire effluent zone in which the suspected amine emerges, trace amounts of it may be recognized. In the present investigation, 2,2'-dithiobis(ethylamine), the amine derived from cystine, was identified in this way. The appropriate effluent zones from columns of Amberlite CG-50 were chromatographed on paper, and the sheets were sprayed with a nitroprusside reagent[12] which is specific for certain sulfur-containing amino acids and amines. *o*-Tyramine was similarly identified in the urine of one normal child on monoamine oxidase blockade, although it could not be detected in the urines of 21 children on monoamine oxidase blockade studied by paper chromatography alone[10].

DISCUSSION

Sixteen aromatic and heterocyclic amines which are usually present in the urines of normal children have been identified in this investigation. Fifteen of these had been detected in a previous study[2] by means of paper chromatography. The present methods have identified 1-methylhistamine, which before had been listed as an unidentified amine. The occurrence of this amine in human urine has been reported by SCHAYER[13]. The tentative identification of kynuramine in urine[2] has been strengthened by the present investigation. The amine present in urine is eluted from Amberlite CG-50 at the same volume as authentic kynuramine, has R_F values on paper that are identical with those of the authentic compound in 4 different solvents, and gives the same color reactions with dimethylaminocinnamaldehyde and with the Ekman spray reagent[12]. The identification of *p*-hydroxybenzylamine and of 3-methoxy-4-hydroxybenzylamine, first reported by KAKIMOTO AND ARMSTRONG[5], has been confirmed.

Of the 16 identified aromatic and heterocyclic amines found in children's urine, synephrine, 3-methoxy-4-hydroxybenzylamine, and *p*-methoxybenzylamine appear to be of exogenous origin, and are eliminated from the urine when children are fed a plant-free diet. Bufotenin and kynuramine are usually detectable only after the administration of monoamine oxidase inhibitors. In the urines examined during this study, *o*-tyramine was detected in one urine, and benzylamine in another. The latter is present in all amine extracts of urines from subjects receiving nialamide. This drug is excreted in the urine; and when urine is hydrolyzed during preparation of amine extracts, the benzylamine moiety of nialamide is freed. In addition to the 16 identified

aromatic amines found in children's urine, another 10 unidentified bases, presumably aromatic amines, were present in the effluents from columns.

Ten aliphatic amines were identified in most or all of the urines studied, as well as an additional 10 bases which could not be identified, but which are probably aliphatic amines. The latter 10 unidentified amines were present in the urine of a normal child who had been placed on a plant-free diet and given neomycin by mouth, and it is reasonable to assume that most or all of them are of endogenous origin.

In an earlier study in which paper chromatography alone had been used[2], it was possible to identify only ethanolamine, pyrrolidine, ethylamine, and β-hydroxypropylamine of the aliphatic group. In the present investigation, methylamine, dimethylamine, and piperidine have also been demonstrated in urine. Their excretion in human urine has been established by other investigators[4,14-16]. The earlier tentative identification[2] of β-hydroxypropylamine has been more solidly established in the present study. Not only does the compound from urine migrate on paper chromatograms at the same rate as the authentic amine in 4 different solvents, but it emerges from columns of Amberlite CG-120 at the same effluent volume.

Putrescine, cadaverine, and 2,2'-dithiobis(ethylamine) have not been reported as constituents of normal urine, although the first two diamines are known to be excreted by cystinurics[17]. The identification of these three diamines in the present investigation is based on identical migration of the urinary compounds and the authentic amines on paper in 4 different solvents, as well as elution from columns of Amberlite CG-50 at the same effluent volumes. In addition, the 2,2'-dithiobis(ethylamine) from urine gave the expected color reaction with nitroprusside.

Efforts to identify the 10 unknown compounds listed in Table V have been unsuccessful. Their elution volumes from Amberlite CG-120 and their migration rates on paper fail to match those of any of a large number of authentic aliphatic amines. It was felt that some of them might be N-acetylated diamines, or the decarboxylation products of the amino acids, glutamine and asparagine, for which authentic samples were unavailable. For this reason, 6 of these unidentified amines (Compounds 51, 52, 53, 55, 56, and 57) were hydrolyzed in 2 *N* hydrochloric acid at 110° for 5 h. Under these conditions, N-acetylhistamine and N-acetylethylenediamine were almost completely hydrolyzed to histamine and ethylenediamine respectively. No change was produced in Compounds 53, 55 and 56 by acid hydrolysis. New ninhydrin-reactive substances were produced by the hydrolysis of Compounds 51, 52 and 57. These were neither the identifiable diamines which might have been expected had the parent compounds been N-acetylated diamines, nor were they β-alanine or γ-aminobutyric acid, as might have been expected had the parent compounds been the amines derived from asparagine or glutamine. It is possible that some of the unidentified ninhydrin-reactive bases found in urine may be decarboxylated simple peptides.

No attempt has been made in this investigation to assess the physiological significance of the wide variety of amines found in human urine. Since most of these continue to be excreted even after reduction of the bacterial flora of the intestine and elimination of plant foods from the diet, it seems likely that they represent intermediate or end points in important metabolic processes. The identity of the unknown amines constantly present in urines should be determined, and the physiological role of these, as well as of many of the identified amines, should be worked out. The techniques here described should be valuable in achieving this end. Not only

may clues to the chemical nature of an unidentified substance be obtained from its point of emergence from the ion exchange column, but the procedure no doubt may also be scaled up to permit isolation in sufficient quantity for chemical identification. In the meantime, knowledge about the normal pattern of excretion of amines in human urine can serve as a useful standard against which to compare the situation encountered in various metabolic diseases.

The methods described here for the identification of amines in urine should be applicable to the study of other physiological fluids and tissue extracts. It is likely that these will be found to contain only a fraction of the number of amines listed in Tables II and III. In such a case, ion exchange column chromatography may accomplish complete separation of most of the amines present.

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SUMMARY

A technique has been developed by which complex mixtures of amines can be separated by column chromatography on the ion exchange resins Amberlite CG-50 and Amberlite CG-120. Identification of amines is made on the basis of the speed of their elution from the resin column, as well as by subsequent paper chromatography of effluent fractions. The method can be applied to amines derived from various physiological fluids, and offers the possibility of isolating unidentified compounds for chemical characterization.

Using the combined technique of ion exchange column chromatography followed by paper chromatography, 26 amines were identified in human urine. These were: methylamine, dimethylamine, ethylamine, ethanolamine, β-hydroxypropylamine, pyrrolidine, piperidine, putrescine, cadaverine, 2,2′-dithiobis(ethylamine), histamine, N-acetylhistamine, 1-methylhistamine, *p*-hydroxybenzylamine, 3-methoxy-4-hydroxybenzylamine, *p*-tyramine, *m*-tyramine, octopamine, synephrine, 3-methoxytyramine, normetanephrine, metanephrine, tryptamine, serotonin, bufotenin, and kynuramine. An additional 20 unidentified bases, presumably amines, were found to be regularly excreted in urine.

Evidence is presented supporting the identification of kynuramine, β-hydroxypropylamine, putrescine, cadaverine, and 2,2′-dithiobis-(ethylamine). These 5 amines have not previously been considered to be constituents of normal human urine.

It is suggested that study of the urinary excretion of amines may provide useful clues as to the mechanisms involved in various metabolic disorders, including those characterized by mental dysfunction.

REFERENCES

[1] W. POLLIN, P. V. CARDON, JR. AND S. S. KETY, *Science*, 133 (1961) 104.
[2] T. L. PERRY, K. N. F. SHAW, D. WALKER AND D. REDLICH, *Pediatrics*, 30 (1962) 576.
[3] N. KIRSCHNER AND McC. GOODALL, *J. Biol. Chem.*, 226 (1957) 207.
[4] K. BLAU, *Biochem. J.*, 80 (1961) 193.
[5] Y. KAKIMOTO AND M. D. ARMSTRONG, *J. Biol. Chem.*, 237 (1962) 208.
[6] C. W. H. HIRS, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 493.

7 S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, 30 (1958) 1185.
8 W. A. Schroeder, R. T. Jones, J. Cormick and K. McCalla, *Anal. Chem.*, 34 (1962) 1570.
9 S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 907.
10 T. L. Perry, *Science*, 136 (1962) 879.
11 T. L. Perry, *Science*, 139 (1963) 587.
12 I. Smith, *Chromatographic and Electrophoretic Techniques*, Vol. I, 2nd edition, William Heinemann, London, 1960.
13 R. W. Schayer, *Physiol. Rev.*, 39 (1959) 116.
14 U. S. von Euler, *Acta Pharmacol.*, 1 (1945) 29.
15 F. Georgi, C. G. Honegger, D. Jordan, H. P. Rieder and M. Rottenberg, *Verhandl. Naturforsch. Ges. Basel*, 70 (1959) 147.
16 A. M. Asatoor, *J. Chromatog.*, 4 (1960) 144.
17 M. D. Milne, A. Asatoor and L. W. Loughbridge, *Lancet*, 1 (1961) 51.

THE COMPARISON OF VARIOUS EVALUATION METHODS FOR PAPER CHROMATOGRAMS OF DERIVATIVES OF HIGHER FATTY ACIDS*

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During our study of the fractions of cholesteryl esters and of glycerides in human sera in normal and in pathological conditions, paper chromatography was used. Our system permits differentiation between a series of cholesteryl esters and one of triglycerides. Various methods of detection were examined, which might also lead to quantitative estimation of the saturated and unsaturated derivatives by means of direct photometry.

MATERIALS AND METHODS

The isolation of cholesteryl esters and of glycerides was performed on columns of aluminum oxide. Direct chromatography of the serum extracts was not used, because the spots of some cholesteryl esters and of glycerides are overloaded.

5 ml of serum were extracted with a mixture of ethanol and ethyl ether (1:3) and the extract was dried under nitrogen. The lipids were re-extracted with light petroleum (three times with 5 ml) and applied to the Al_2O_3 column. Cholesteryl esters were eluted with 50 ml of carbon tetrachloride and glycerides with 50 ml of chloroform.

Both fractions were evaporated to dryness under nitrogen. The ester fraction was dissolved in 3 ml of chloroform and the fraction of glycerides in 0.5 ml of chloroform. The solutions were applied in amounts of 50 μl on sheets of Whatman No. 3 paper impregnated with paraffin oil[1].

As the mobile phase, a mixture of acetic acid–chloroform–paraffin oil (80:15:5; v/v/v) was used.

After chromatography with this mixture at room temperature the chromatograms were dried at 80–100° and detected by dipping in 1% aqueous potassium permanganate, then immediately washed in running water. The spots were brown on the white or slightly brownish background.

* A part of this paper was presented at the Conference on Paper Chromatography, Prague, June 21–22, 1961.

Fractions of cholesteryl esters and glycerides of normal human sera were used as reference standards. These fractions were isolated on an Al_2O_3 column as described above. These "normal" extracts were applied on the paper in amounts of 30, 50, 70 and 100 μl.

After detection the chromatograms of the standard mixtures were evaluated:

A. In transmitted light; B. In reflected light; C. In transmitted light, after impregnation of the chromatograms with paraffin oil; D. By microphotometry of the negatives of the chromatograms on photographic film.

In all four cases, the densitometric (photometric) curves were registered automatically and evaluated both by measurement of the areas (five times using a polar planimeter; expressed in cm^2) and by measurement of the maximum density (peak heights in mm) of the curves.

In the first three cases the green filter from the Lange colorimeter was used. In cases A and C the densitometer "Chromatometer 3c" (B. Lange, Berlin) and in case B a reflecting densitometer of our own construction[2] was used. Registration was carried out using a mirror galvanometer (Multiflex MG 2, B. Lange) and a recording apparatus ("Nachlaufschreiber 2", B. Lange). Microphotometry was performed with a Czechoslovak recording microphotometer (Keramos, Brno).

The measured values were linearized as a semilogarithmic dependence between the maximum density or the area and the logarithm of the applied volume,

$$y = a + bx,$$

where y = maximum density in mm or area of the curve in cm^2,
x = logarithm of the volume v (μl) of the standard mixture ($x = \log v$),
a, b = constants of the calibration equation.

Semilogarithmic linearization has already been used in the photometric evaluation of chromatograms of many other substances[3] and in spot-tests[4].

The calibration equations were calculated by means of the least-square method.

From the differences between measured and calculated values the standard deviation of y, s_y, was calculated. In order to compare more closely the deviations for various derivatives, the interval Δ_v, corresponding to s_y in the mean value of x ($\bar{v} = 56.9$ μl), was calculated. The scheme of the calculation procedure is demonstrated in Fig. 1.

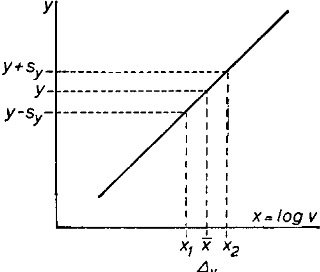

Fig. 1. Comparison of the accuracy of the calibration equations. s_y = Standard deviation of y from the equation $y = a + bx$; $x = \log v$. $s_y = \pm \sqrt{\frac{(y - \tilde{y})^2}{n - 2}}$; y = Measured value (cm^2 or mm), $\tilde{y}$ = Value calculated from calibration equation. v = Volume of standard mixture, in μl. $\bar{x}$ = Average value of x (corresponding value $v = 56.93$ μl). Δ_v = Interval corresponding to the values $Y + s_y$ and $Y - s_y$.

RESULTS AND DISCUSSION

At the beginning of this work, various methods were tried for separating and detecting saturated and unsaturated derivatives of the higher fatty acids. The methods examined did not give any satisfactory results for the saturated derivatives. The derivatives of unsaturated higher fatty acids (UHFA) were then examined. Densitograms of the chromatograms of glycerides of UHFA showed, however, that the chromatographic method used gave incomplete separation of these derivatives. In addition, the detection method proved unsuitable for quantitative analysis. None of the four methods mentioned gave results of a sufficient degree of accuracy due probably to lack of homogeneity in the coloration of the spots and of the background. The base line showed great variability along the length of the chromatograms. Because of this, the methods of analysis (mainly the detection) must be further perfected, and the results presented are valid only for the cholesteryl esters of UHFA.

The densitometric curves obtained with the four methods of photometry mentioned, evaluated according to their areas and according to their maximum densities, are summarized in Table I.

The relation between the measured values and the logarithms of the applied volumes is shown in Fig. 2.

The results achieved are to some extent surprising. Theoretically (*cf.* FALTA[5]), it could be presumed that the best results would be obtained by photometry of the impregnated chromatograms in transmitted light. However, this method was shown to be less accurate than the other three. The reason for this possibly lies in the

TABLE I

RESULTS OF THE DENSITOMETRY OF CHROMATOGRAMS OF CHOLESTERYL ESTERS

Method*	Ester**	*Volume of the standard mixture (µl)*				*Volume of the standard mixture (µl)*			
		30	*50*	*70*	*100*	*30*	*50*	*70*	*100*
		Height of peaks, in mm (maximum density)				*Area of densitograms, in cm² × 5*			
	O	20	45	57	63	6.5	20.3	24.9	29.8
A	L	69	88	99	93	40.0	60.3	72.0	71.0
	A	29	42	59	63	11.0	17.1	21.9	22.9
	O	33	50	60	83	15.2	25.2	36.5	45.7
B	L	70	109	132	150	43.4	73.1	97.3	114.8
	A	33	57	76	93	13.4	24.5	27.3	32.5
	O	18	24	46	56	4.1	6.7	18.4	22.3
C	L	64	74	132	162	30.4	43.8	68.9	90.9
	A	37	17	60	78	9.7	2.1	23.0	24.2
	O	27	45	67	78	10.5	19.3	31.8	36.4
D	L	75	89	153	174	35.9	59.6	92.5	116.4
	A	29	42	80	96	8.4	11.7	28.7	35.2

* A = Densitometry in transmitted light without impregnation of chromatograms; B = Densitometry in reflected light; C = Densitometry in transmitted light; chromatograms impregnated with paraffin oil; D = Microdensitometry of photographic negatives of the chromatograms.

** Abbreviations: O = cholesteryl oleate; L = cholesteryl linoleate; A = cholesteryl arachidonate.

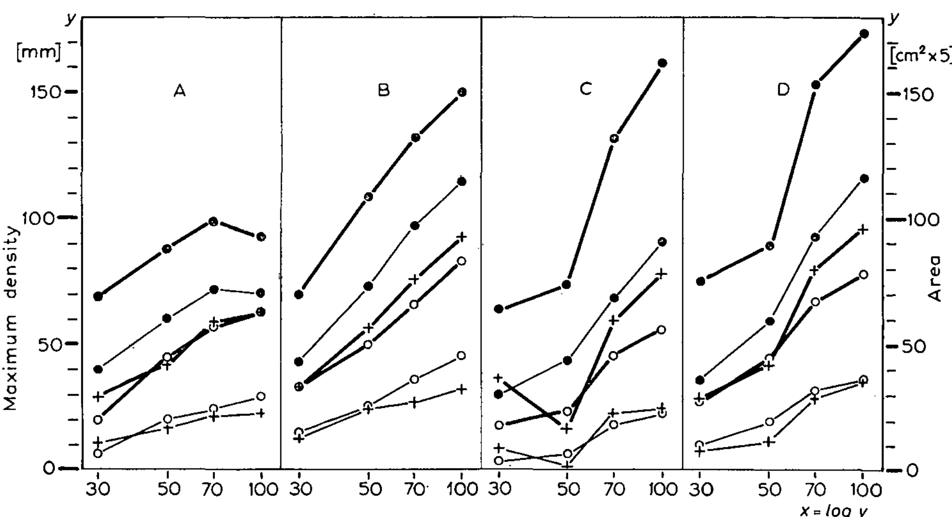

Fig. 2. Results of the photometry of chromatograms of cholesteryl esters. y = Measured photometric values; height of peaks in mm (measurement of maximum density) or area in $cm^2 \times 5$ (measurement of area enclosed by densitometric curves). x = Logarithm of volume of standard mixture applied to chromatograms. Thick lines = measurement of maximum density. Thin lines = measurement of area. Methods of densitometry are indicated by large capitals. Cholesteryl esters: O——O oleate; ●——● linoleate; +——+ arachidonate.

variation in texture of the paper used, which is more evident in transmitted light than in reflected light. The thinner papers (Whatman No. 4 and No. 1) are more homogeneous, but the separation was considerably worse than on Whatman No. 3 paper.

The impregnation procedure carried out prior to densitometry did not affect the colour of the detection reaction. The impregnated chromatograms were registered, extracted with light petroleum and registered again. The resulting densitograms were practically identical with densitograms obtained prior to impregnation.

From Fig. 2 it is evident that method B (measurement of reflectance) is better than the other three methods. This figure alone, however, cannot determine whether it is more accurate to evaluate the densitometric curves according to their areas or according to their maximum densities (*i.e.* peak heights). For this reason we calculated

TABLE II

CALIBRATION EQUATIONS FOR THE EVALUATION OF CHROMATOGRAMS OF CHOLESTERYL ESTERS IN REFLECTED LIGHT

$y = a + bx$; $x = \log v$. y in mm (measurement of maximum density) or $cm^2 \times 5$ (measurement of area).

Ester*	Measurement of maximum density				Measurement of area			
	a	b	s_y (mm)	Δ_v (µl)	a	b	s_y ($cm^2 \times 5$)	Δ_v (µl)
O	−110.30	95.88	±2.48	6.80	−73.48	59.34	±1.75	7.75
L	−155.95	154.50	±3.77	6.39	−161.76	138.96	±2.30	4.33
A	−138.42	115.75	±1.12	2.64	−38.18	35.67	±1.74	11.63

* O = cholesteryl oleate; L = cholesteryl linoleate; A = cholesteryl arachidonate. Other explanations in text.

the calibration equations (the least-square method), s_y and corresponding values Δ_v for both the measurement of areas and the measurement of maximum densities. The results, shown in Table II, demonstrate the greater accuracy obtained by measuring the maximum density. This method is also much faster than measuring the areas.

On the basis of these results we were able to evaluate the chromatograms of derivatives of UHFA from various sera. The data obtained (some of which have been published[6]) agree with those given in the literature.

The calculated values in μl were converted to weight units or to relative concentrations in the following manner:

If c_s is the concentration of the particular derivative in the standard mixture, then its weight amount in volume v is w:

$$w = v.c_s,$$
and $$\log w = (y - a)/b + \log c_s,$$
or $$\log w = (y - a')/b,$$
where $$a' = a - b \log c_s.$$

If the actual concentration is unknown, we can use the relative concentration (in %). In this case we obtain the results in terms of the relative concentrations.

Thus ZÖLLNER AND WOLFRAM[7] found the relative concentrations of cholesteryl esters in human serum to be:

oleate	28.4 %
linoleate	57.1 %
arachidonate	14.5 %.

In this manner we calculated from densitograms (evaluating the reflectance records according to their maximum densities) the concentrations of cholesteryl esters in sera of man and of various animals. Our results conform with the data of other authors obtained by other methods.

The method of photometry used, *i.e.* the photometric evaluation of chromatograms in reflected light, has four main advantages.

(1) It is possible to use thick paper (Whatman No. 3), which is more homogeneous in reflected than in transmitted light.

(2) The method is quick. With our densitometer[2] it is possible to record more than a hundred chromatograms per day.

(3) The method of evaluating the densitograms according to their maxima is faster than measuring areas, and, moreover, in our experiments this method proved to be more accurate than the latter.

(4) Measurement of maxima also allows the evaluation of chromatograms with incomplete separation of the spots.

SUMMARY

Paper chromatography was used in the determination of derivatives of higher fatty acids from biological material. The optimum conditions for quantitative evaluation by photometry of chromatograms were investigated.

The method used did not give any satisfactory resolution of saturated derivatives

and the results of the densitometry of the chromatograms of glycerides demonstrated that the method of chromatography and detection did not permit quantitative evaluation.

Cholesteryl esters of unsaturated fatty acids (cholesteryl oleate, linoleate and arachidonate) were separated to a sufficient degree.

For quantitative evaluation we compared four methods: (1) direct photometry of chromatograms in transmitted light, (2) direct photometry in reflected light, (3) photometry of chromatograms impregnated with paraffin oil, and (4) microphotometry of the photographic negatives of chromatograms.

The resulting densitograms were evaluated (a) according to the areas and (b) according to the maximum density of the curves. Of these four methods, direct photometry in reflected light with evaluation according to the maximum density gave the most accurate results.

The analytical procedure mentioned for the estimation of cholesteryl esters in sera gave results conforming with those obtained by other methods given in the literature.

REFERENCES

1 Č. MICHALEC AND J. STRÁŠEK, *J. Chromatog.*, 4 (1960) 254.
2 D. HAL'AMA, *Chem. Zvesti*, 13 (1959) 254.
3 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New York, 1961.
4 D. HAL'AMA, in *Some General Problems of Paper Chromatography*, Publishing House of Czech. Acad. Sci., Prague, 1962, pp. 205–210.
5 W. FALTA, in *Jenaer Jahrbuch 1955*, 2. Teil, VEB G. Fischer, Jena, 1955, pp. 212–246.
6 Č. MICHALEC, M. ŠULC, J. MĚŠTAN, D. HAL'AMA AND V. KOMAN, *Sb. Lékař.*, 63 (1961) 99.
7 N. ZÖLLNER AND G. WOLFRAM, *Klin. Wochschr.*, 39 (1961) 817.

FLUORESCENT DETECTION AND DETERMINATION OF ORGANIC COMPOUNDS

III. CARBONYL DERIVATIVE SEPARATION BY CHROMATOGRAPHY

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INTRODUCTION

Since carbonyl compounds are widespread in both natural products and air impurities, much research has been devoted to the aim of developing a rapid method of separation and identification of aromatic and aliphatic carbonyl compounds, both saturated and unsaturated, on the semimicro and micro scale.

The most often applied method for derivatization of carbonyl compounds is the formation of 2,4-dinitrophenyl (DNP) hydrazones. The chromatography of small amounts of these materials has been attempted on both untreated[1-3] and treated papers[4-7]. The latter method, that of reversed phase chromatography, has generally been more successful. R_F values have been reported for homologous members of aliphatic aldehyde 2,4-DNP-hydrazones from C_1 to C_6 on propylene glycol treated paper[7], and for the C_7 to C_{11} derivatives on vaseline-treated paper. Excellent separations were obtained using paraffin as a paper impregnant for DNP-hydrazones of lower aliphatic carbonyls[8] with the results expressed relative to formaldehyde DNP-hydrazone movement. Paper impregnated with sodium bisulfite was used to separate carbonyl compounds due to differential rates of reaction[9] followed by location with DNP-hydrazine spray.

Other carbonyl derivatives separated on paper include Girard P and T[10], benzene sulfohydroxamates[11] and cyanoaceto hydrazones[12].

The application of vaseline-treated paper to separate carbonyl derivatives has previously been reported from this laboratory[13]. The rapidity of the reaction of 2-diphenylacetyl-1,3-indandione-1-hydrazone[14] with carbonyls, the ease of purification, the high melting points of the derivatives (azines) and the ease and sensitivity of detection have led to this further study on the separation and detection of these compounds[15].

EXPERIMENTAL

A "Thomas-Kolb" jar for 10 in. × 10 in. paper was used for ascending and a round tank equipped with frame and trough was used for descending chromatography. The

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tanks were lined with Whatman No. 1 paper saturated with the developing solvent. Methanol–water (12:1) was used as a developing solvent for Whatman No. 1 paper impregnated with vaseline ("Blue Seal" Chesebrough-Ponds, Inc., New York, N.Y.).

Reagent grade solvents and single distilled water were used. The carbonyl compounds were reagent grade obtained from commercial sources and used without purification. The azines were prepared as previously reported[13] from 2-diphenylacetyl-1,3-indandione-1-hydrazone and were recrystallized from chloroform–methanol until a constant melting point compound was obtained (Kofler Hot Stage, corrected).

PROCEDURE

The papers were impregnated by rapidly drawing them through a glass tray containing 7% w/v of vaseline in petroleum ether (30–60°, fraction). Each solution was immediately used for two papers. Stock solutions of the azine and the hydrazone were prepared in chloroform at 1 mg/ml. Samples for chromatography of 2 μl were applied from 1 mg/ml solutions. The average time for ascending chromatography was 6 h for an 8 in. front, and for descending, 6 h for a 10 in. front. The spots were located by their fluorescence using a 3660 Å ultraviolet hand lamp (Mineralite Ultraviolet Products, Inc., San Gabriel, Calif.).

RESULTS AND DISCUSSION

Various combinations of polar and non polar solvents for development of chromatograms on untreated and vaseline-treated paper were used. In early experiments ethyl ether was used as a vaseline solvent but petroleum ether as a solvent resulted in more even treatment of the paper. Developing systems on untreated paper gave separation from the reagent, but were not successful in the resolution of homologous members. The R_F values of various azines using both treated and untreated papers are shown in Table I.

R_F values for methyl ketone azines from C_3 to C_{11} are shown in Table II. The

TABLE I

R_F VALUES OF CARBONYL AZINES ON TREATED AND UNTREATED PAPER

Azine	R_F[a]	
	Vaseline-treated[b]	*Untreated*[c]
Acetaldehyde	0.74	0.53
Citraldehyde	0.58	0.57
Vanillin[d]	0.75	0.02
Veratraldehyde[d]	0.80	0.06
Piperonal[d]	0.67	0.14
Salicylaldehyde	0.71	0.21
Benzophenone	0.57	0.58

[a] 25° ± 2°.
[b] Descending, methanol–water (12:1), 6 h for 10 in. front.
[c] Descending, hexane saturated with water, 2 h for 10 in. front.
[d] Previously unreported derivatives.

TABLE II

R_F VALUES FOR METHYL KETONE AZINES ON TREATED PAPER: ASCENDING DEVELOPMENT

Azine of methyl ketone	R_F[a]	R_F *mixture*
C_3	0.80	—
C_4	0.83	0.81
C_5	0.79	0.76
C_6	0.75	0.71
C_7	0.68	0.66
C_8	0.59	0.59
C_9	0.55	0.52
C_{10}	0.48	0.46
C_{11}	0.40	0.39

[a] Methanol–water (12:1), 6 h for an 8 in. front at 25°. Average of five determinations.

average deviations are ± 0.04 for C_3 to C_7 and ± 0.02 R_F units for C_8 to C_{11} for five separate vaseline-treated papers. The deviations between duplicates on the same sheet are less, indicating variations in the impregnation process between separate papers. Treatment with 14% w/v vaseline slowed development time, the front moving only 5 in. in 24 h for ascending chromatography. R_F values were lower and no better separation was obtained for the aliphatic series. A mixture of 1 ml of 1 mg/ml each of the C_3 to C_{11} azines was combined and evaporated *in vacuo* to 1 ml and an aliquot was chromatographed. The results correspond to those of the separate azines (Table II) but with only one spot at the C_3–C_5 position. The C_4 azine was mixed with an equal amount of each of the C_5 to C_9 azines and successfully separated as compared to the pure azines in an adjacent lane on the same treated paper (Table III).

By a sequential decrease in factors of 10 of the concentration of the samples chromatographed, the lower limit range of detection was found to be between 0.02–0.2 μg. 2-Hexanone and 2-decanone azines were used as representative compounds and the R_F values corresponded to ± 0.02 units to those in Table II.

A modification of the rapid derivatization technique of these authors[13] was attempted first at 10 μg/ml and then at 1 μg/ml for reaction detection. A drop of the hydrazone (at 1 mg/ml) and a drop of hydrochloric acid were added to the carbonyl in chloroform and the mixture was heated. This was then rapidly evaporated *in vacuo*

TABLE III

R_F VALUES OF AZINE PAIR MIXTURES: ASCENDING DEVELOPMENT

Azine of methyl ketone	R_F[a]	R_F *pure azine adjacent lane*
$C_4 + C_5$	0.83 0.79	C_4 0.83
$C_4 + C_6$	0.83 0.75	C_5 0.77
$C_4 + C_7$	0.82 0.67	C_6 0.75
$C_4 + C_8$	0.83 0.62	C_7 0.66
$C_4 + C_9$	0.83 0.56	C_8 0.61
		C_9 0.55

[a] Methanol–water (12:1), 25°.

to approximately 0.1 ml, and 1 μl was applied and chromatographed. At the 10 μg/ml level using benzaldehyde, benzophenone and 2-heptanone, only benzaldehyde gave the same R_F value as its previously prepared azine derivative (0.69 ascending, using methanol–water, 12:1). The two other carbonyls had R_F values of 0.79 and 0.80. A faint yellow spot was observed at 0.83 for the hydrazone treated in the same manner. This must be interpreted as being a reaction between a carbonyl present in the solvent and the reagent. The reaction indicated with benzaldehyde probably is due to the greater reactivity of the unhindered aromatic azine as compared to the carbonyl present in chloroform. This confirms an earlier report that the aromatic compounds are more reactive than the aliphatic[1]. At a carbonyl concentration of 1 μg/ml, R_F values were found only at 0.80 to 0.83 corresponding to an acetone impurity in the chloroform.

Chromatography of some carbonyl group and hydrazone reaction solutions was attempted in order to observe any interference in the detection of carbonyl compounds. Although no definite conclusions were reached, spots obtained from these solutions are different from those of carbonyl derivatives with regard to their resolution and their pale yellow fluorescence in ultraviolet light. However, these materials may be contaminated with aldehydes or ketones or they have reacted with the hydrazone to form other fluorescent products than azines.

TABLE IV

COMPARISON OF AZINE AND 2,4-DINITROPHENYLHYDRAZONE MELTING POINTS OF METHYL KETONES

Compound	*M.p. of azine*	*Δ m.p. with increase in -CH₂-*	*M.p. of 2,4-DNPH*	*Δ m.p. with increase in -CH₂-*
2-Propanone	226–227	—	126	—
2-Butanone	197.5–198	—29	116–117	— 9
2-Pentanone	166–167	—21	143–144	+27
2-Hexanone	135–136	—30	110	—34
2-Heptanone	147–148.5	+12	89	—21
2-Octanone	128.5–130.5	—18	58	—30
2-Nonanone	126.5–127.5	— 3	—	—
2-Decanone[a]	121–122	— 5	—	—
2-Undecanone	105–106	—16	—	—

[a] New compound

A comparison of the 2,4-DNP-hydrazone melting points[16] to those of the azine methyl ketones (Table IV) shows that the higher aliphatic azines (C_7 to C_{11}) have distinct melting points while the hydrazones are low melting or oils.

CONCLUSION

The 2-diphenylacetyl-1,3-indandione-1-azines must rank as one of the better derivatives for carbonyl determination. Their distinct R_F values, melting points and high order of formation may eventually lead to these derivatives replacing some of the presently more familiar non-fluorescent derivatives.

SUMMARY

R_F values of various aliphatic and aromatic fluorescent 2-diphenylacetyl-1,3-indandione-1-azines of carbonyl compounds were given for vaseline-treated and untreated paper. The melting points of the C_3–C_{11} methyl ketone azines were contrasted to the corresponding 2,4-dinitrophenylhydrazones and found in the C_7–C_{11} compounds to be more distinct.

REFERENCES

[1] N. D. CHERONIS AND V. M. LEVEY, *Microchem. J.*, 1 (1957) 228.
[2] D. A. FORSS, E. A. DUNSTONE AND W. STARK, *Chem. Ind. (London)*, (1954) 1292.
[3] R. G. RICE, G. J. KELLER AND J. G. KIRCHNER, *Anal. Chem.*, 23 (1951) 194.
[4] D. F. MEIGH, *Chem. Ind. (London)*, (1956) 986.
[5] F. KLEIN AND K. DE JONG, *Rec. Trav. Chim.*, 75 (1956) 1285.
[6] E. SUNDT AND M. WINTER, *Anal. Chem.*, 30 (1958) 1620.
[7] R. ELLIS, A. M. GADDIS AND G. T. CURRIE, *Anal. Chem.*, 30 (1958) 475.
[8] A. M. ASATOOR, *J. Chromatog.*, 7 (1962) 415
[9] A. G. NEWCOMBE AND S. G. REID, *Nature*, 172 (1953) 455.
[10] R. B. SELIGMAN, M. D. EDMONDS, A. E. O'KEEFE AND L. A. LEE, *Chem. Ind. (London)*, (1954) 1195.
[11] H. UNO AND A. KOYAMA, *J. Ferment. Technol. (Japan)*, from *C.A.*, 47 (1953) 1008b.
[12] J. FRANC AND G. CELIKOVSKA, *Collection Czech. Chem. Commun,*, 26 (1961) 667, from *J. Chromatog., Chromatog. Data*, 7 (1962) D41.
[13] R. BRANDT, J. C. KOUINES AND N. D. CHERONIS, *Microchem. J.*, 6 (1962) 519.
[14] R. BRANDT AND N. D. CHERONIS, *Microchem. J.*, 5 (1961) 110.
[15] J. C. KOUINES, *Master's Thesis*, Brooklyn College, June 1962.
[16] N. D. CHERONIS AND J. B. ENTRIKIN, *Semimicro Qualitative Organic Analysis*, 2nd Ed., Interscience, New York, 1957, p. 663.

IDENTIFIZIERUNG ORGANISCHER VERBINDUNGEN*

LIV. MITTEILUNG. PAPIERCHROMATOGRAPHISCHE TRENNUNG UND IDENTIFIZIERUNG FOTOGRAFISCHER ENTWICKLERSUBSTANZEN

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In dieser Arbeit wollen wir eine Methode für Trennung und Identifizierung der Stoffe die man als Entwicklersubstanzen in verschiedenen Handelsprodukten der Entwickler benützt (Tabelle I), beschreiben. Es handelt sich insgesamt um verschieden substituierte aromatische Hydroxy- oder Aminostoffe. Zu den geprüften Substanzen haben wir auch N,N-Diäthyl-*p*-phenylendiamin und N-Äthyl-N-oxyäthyl-*p*-phenylendiamin, die ursprünglich in der Farbfotografie, in letzter Zeit aber auch in der Schwarzweissfotografie mit Vorteil benützt werden[1], zugefügt. Durchwegs handelt es sich um unbeständige Substanzen, die leicht oxydiert werden können. Diese Eigenschaft, die

TABELLE I

ÜBERSICHT DER GEPRÜFTEN SUBSTANZEN

Nr.	*Verbindung*	*Handelsname*
1	1,2-Dioxybenzol	Brenzcatechin, Pyrokatechin, Cachin, Katchin, Elconal
2	1,4-Dioxybenzol	Hydrochinon, Quinol
3	1,2,3-Trioxybenzol	Pyrogallol, Pyro, Pyral
4	1-Chlor-2,5-dioxybenzol	Chlorhydrochinon, Adurol Hauff.
5	1-Brom-2,5-dioxybenzol	Bromhydrochinon, Adurol Schering.
6	1-Phenyl-pyrazolidon-3	Phenidon
7	1-Oxy-2-aminobenzol	*o*-Aminophenol
8	1-Oxy-4-aminobenzol	*p*-Aminophenol, Rodinal, Unal
9	1-Oxy-4-(N-methyl-amino)-benzol	Metol, Adilol, Elon
10	N-(4-Oxyphenyl)-aminoessigsäure	Glycin, Iconyl, Kodurol
11	1-Oxy-2-(N-oxyäthyl-amino)-benzol	Atomal
12	1,2-Diaminobenzol	*o*-Phenylendiamin
13	1,4-Diaminobenzol	*p*-Phenylendiamin, Diamin, Paramine
14	N.N-Diäthyl-1,4-diaminobenzol	N,N-Diäthyl-*p*-phenylendiamin, TSS, Gevadiamin
15	N-Äthyl-N-oxyäthyl-1,4-diaminobenzol	N-Äthyl-N-oxyäthyl-*p*-phenylendiamin, T 32
16	1-Oxy-2,4-diaminobenzol	Amidol, Diamol.

* LIII. Mitteilung: *Mikrochim. Acta*, (1962) 1137.

einerseits die Arbeit kompliziert, erleichtert uns andererseits die Sichtbarmachung mittels verschiedener Farbreaktionen.

Für die Trennung und Identifizierung der Entwicklersubstanzen ist die Papierchromatographie besonders gut geeignet. Für diesen oder ähnlichen Zweck haben schon einige Autoren[2-5] diese Methode benützt. Sie verwendeten dazu die unvorbehandelten Papiere und ein Gemisch von *n*-Butylalkohol, Essigsäure und Wasser als bewegliche Phase. Beim Nachprüfen dieser Methoden erhielten wir nicht die entsprechenden Resultate; die Trennung war nicht ausreichend und manchmal wurden gezogene Flecken gebildet, besonders bei solchen Stoffen, die in Form der Salze mit anorganischen Säuren aufgetragen werden. In einigen Fällen trat dazu die Zersetzung einiger chromatographierten Stoffe.

Das Problem der Trennung dieser Substanzengruppe wurde von uns durch Verwendung einerseits von neutralen oder schwach basischen Lösungsmittelsystemen mit Formamid als stationäre Phase und Hexan, Benzol, Tetrachlormethan, Chloroform und Äthylacetat oder deren Mischungen mit eventueller Anwesenheit von Pyridin oder Ammoniak als bewegliche Phase, andererseits von sauren Systemen auf unvorbehandelten Papieren mit Äthanol, *n*-Propanol oder *n*-Butanol oder deren Mischungen und Zugabe von Salzsäure als bewegliche Phase gelöst. Für die Identifizierung der *p*-Hydroxyphenylaminoessigsäure wurde als bewegliche Phase ein Gemisch von Äthanol, *n*-Butanol und Kaliumchlorid auf mit Kaliumchlorid imprägnierten Papieren gewählt (Tabelle II).

EXPERIMENTELLER TEIL

Alle Versuche wurden absteigend mit dem Whatmanpapier No. 3 durchgeführt. Die chromatographierten Verbindungen wurden als äthanolische oder äthanolisch-wässrige Lösungen auf die Chromatogramme aufgetragen und bei Raumtemperatur (20–25°) entwickelt. Die verwendeten Chemikalien waren handelsübliche Produkte, Chlor- und Bromhydrochinon wurden nach Angaben der Literatur[6,7] hergestellt. Ammoniak und Salzsäure wurden in Form der handelsüblichen konzentrierten wässrigen Lösungen benützt.

Für Chromatographie auf unvorbehandelten Papieren wurden die Gemische der Alkohole mit Salzsäure verwendet (Tabelle II). Für Chromatographie auf imprägnierten Papieren wurde ein Papierstreifen durch eine 20 %ige äthanolische Formamidlösung gezogen und bei Raumtemperatur 10–20 Minuten getrocknet. Als bewegliche Phase dienten da verschiedene weniger polare Lösungsmittel unter eventueller Zugabe von Pyridin (Tabelle II). In einigen Fällen wurde die Chromatographie in Ammoniakatmosphäre (auf den Boden der Kammer wurden ca. 10 ml Ammoniak eingetragen) durchgeführt. Bei der Chromatographie auf den mit Kaliumchlorid imprägnierten Papieren wurde der Papierstreifen durch eine 5 %ige wässrige Kaliumchloridlösung gezogen und bei Raumtemperatur über Nacht getrocknet. Die bewegliche Phase war in diesem Falle ein Gemisch von 2 Teile Äthanol mit 2 Teilen *n*-Butanol und 1 Teil 5 %iger wässriger Kaliumchloridlösung. So gewonnene Lösung wurde nach Abtrennung von ausgefallenem festem Kaliumchlorid benützt.

Die zum Durchfluss von 20–30 cm erforderliche Laufzeit auf den mit Formamid imprägnierten Papieren ist ungefähr 1–2 Stunden. Auch das Trocknen der Papiere ist in diesem Falle sehr schnell und zwar einige Minuten bei Raumtemperatur. Die

TABELLE II

LÖSUNGSMITTELSYSTEME

Bezeichnung	*Stationäre Phase*	*Bewegliche Phase*
S 1	Formamid	Hexan
S 2	Formamid	Hexan–Pyridin (50:1)
S 3	Formamid	Hexan + Ammoniak
S 4	Formamid	Hexan–Benzol (1:1)
S 5	Formamid	Hexan–Benzol–Pyridin (25:25:1)
S 6	Formamid	Hexan–Benzol (1:1) + Ammoniak
S 7	Formamid	Benzol
S 8	Formamid	Benzol–Pyridin (50:1)
S 9	Formamid	Benzol + Ammoniak
S 10	Formamid	Tetrachlormethan
S 11	Formamid	Tetrachlormethan–Pyridin (50:1)
S 12	Formamid	Tetrachlormethan + Ammoniak
S 13	Formamid	Benzol–Chloroform (1:1)
S 14	Formamid	Benzol–Chloroform–Pyridin (25:25:1)
S 15	Formamid	Benzol–Chloroform (1:1) + Ammoniak
S 16	Formamid	Chloroform
S 17	Formamid	Chloroform–Pyridin (50:1)
S 18	Formamid	Chloroform + Ammoniak
S 19	Formamid	Tetrachlormethan–Äthylacetat (1:1)
S 20	Formamid	Tetrachlormethan–Äthylacetat-Pyridin (25:25:1)
S 21	Formamid	Tetrachlormethan–Äthylacetat (1:1) + Ammoniak
S 22	Formamid	Chloroform–Äthylacetat (1:1)
S 23	Formamid	Chloroform–Äthylacetat–Pyridin (25:25:1)
S 24	Formamid	Chloroform–Äthylacetat (1:1) + Ammoniak
S 25	Formamid	Chloroform–Äthylacetat (1:4)
S 26	Formamid	Chloroform–Äthylacetat–Pyridin (10:40:1)
S 27	Formamid	Chloroform–Äthylacetat (1:4) + Ammoniak
S 28	Formamid	*n*-Propanol–HCl (2:1)
S 29	Formamid	*n*-Propanol–HCl (4:1)
S 30	Formamid	*n*-Propanol–HCl (9:1)
S 31	Formamid	*n*-Butanol–Äthanol–HCl (1:1:1)
S 32	Formamid	*n*-Propanol–*n*-Butanol–HCl (1:1:1)
S 33	Formamid	*n*-Butanol–HCl (5:1)
S 34	Kaliumchlorid	*n*-Butanol–Äthanol–5%iger KCl (2:2:1)

Laufzeit in den Alkohol–Salzsäure Systemen auf den unvorbehandelten Papieren ist wesentlich länger, so dass es vorteilhaft ist, die Chromatographie über Nacht durchzuführen (ungefähr 20 Stunden). Auch das Trocknen der Papiere ist in diesem Falle langsamer und dauert bei Raumtemperatur einige Stunden. Für eine ganze Reihe der Sichtbarmachungen ist aber das vollständige Trocknen der Papiere nicht nötig; es genügt das minimale Abtrocknen. Die R_F-Werte sind in Tabelle III angegeben.

Die Sichtbarmachung wurde auf verschiedenartige Weise durchgeführt, und zwar mit den folgenden Reagenzien:

(A) einem frisch bereiteten Gemisch von 1%iger Kaliumhexacyanoferrat(III)-lösung und 15%iger Eisen(III)-chloridlösung (1:1);

(B) 0.1%iger wässriger 1-Diazo-2-chlor-4-nitrobenzol 1,5-Naphthalindisulfonatlösung[7,8] (B1) und nachfolgendem Besprühen mit 10%iger Kalilauge (B2);

(C) 5%iger wässriger Silbernitratlösung (C1), oder einem Gemisch von 5%iger Silbernitratlösung mit Ammoniak (9:1) (C2);

TABELLE

R_F-WERTE (× 100) DER

Nr.*	Lösungsmittelsysteme**													
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
1	00	01	—	00	10	—	02	28	—	00	09	—	03	26
2	00	00	—	00	01	—	00	06	—	00	01	—	00	05
3	00	00	—	00	00	—	00	02	—	00	00	—	00	02
4	00	00	—	00	05	—	00	24	—	00	05	—	02	18
5	00	00	—	00	06	—	01	27	—	00	06	—	03	21
6	00	00	—	06	10	—	30	37	—	07	12	—	53	59
7	00	00	—	01	08	—	05	26	—	00	08	—	10	27
8	00	00	—	00	01	—	01	05	—	00	01	—	03	07
9	00	00	—	02	08	—	10s	28	—	01	07	—	15s	36
10	00	00	—	00	00	—	00	00	—	00	00	—	00	00
11	00	00	—	00	01	—	01	08	—	00	02	—	02	13
12	01	01	01	08	10	07	26	29	27	09	10	10	36	34
13	00	00	00	01	01	01	04	05	06	00	01	01	11	11
14	00	s	46	s	s	78	s	s	88	s	s	85	s	s
15	00	00	00	00	s	04	s	s	21	00	s	06	s	s
16	00	00	00	00	00	00	00	00	00	00	00	00	00	00

* Bezeichnung der geprüften Substanz, siehe Tabelle I.
** Siehe Tabelle II. s = Streifen.

(D) 1 %iger wässriger Kaliumhexacyanoferrat(III)-lösung (D1) und nachfolgender Wirkung von Ammoniakdämpfen (D2);

(E) 15 %iger wässriger Eisen(III)-chloridlösung;

(F) 3 %iger wässriger Kaliumdichromatlösung (F1) und nachfolgender Wirkung von Ammoniakdämpfen (F2);

(G) Ehrlich-Reagens[9];

(H) Dragendorff-Reagens[9];

(I) Kaliumjodoplatinat[9];

(J) durch Diazotierung (das Chromatogramm wird in eine Kammer eingehängt, auf deren Boden sich mit Salzsäure angesäuertes Natriumnitrit befindet, nach einigen Minuten herausgenommen und 10 Minuten frei an der Luft hängengelassen) (J1), und nachfolgendem Besprühen mit R-Salzlösung (1 %ige Lösung in 5 %iger Sodalösung) (J2), und dann nachfolgendem Besprühen mit 10 % iger Kalilauge (J3);

(K) durch Diazotierung und nachfolgendem Besprühen mit Resorcinlösung (1 %ige Lösung in 5 %iger Sodalösung) (K1), und nachfolgendem Besprühen mit 10 %iger Kalilauge (K2).

Die resultierenden Färbungen sind in Tabelle IV zusammengefasst.

ERGEBNISSE UND DISKUSSION

Aus den erhaltenen Resultaten ist ersichtlich, dass die Papierchromatographie zur Trennung dieser Stoffe geeignet ist. Man kann sie gut auf den mit Formamid imprägnierten Papieren chromatographieren. Die Aminostoffe, besonders mit zwei Aminogruppen kann man auch auf den unvorbehandelten Papieren in stark sauren Systemen in Form der Salze (am besten als Hydrochloride) chromatographieren. Einige dieser Stoffe sind so unbeständig, dass man sie in Form ihrer Salze mit Salz-

III

GEPRÜFTEN SUBSTANZEN

*Lösungsmittelsysteme***																			
S15	*S16*	*S17*	*S18*	*S19*	*S20*	*S21*	*S22*	*S23*	*S24*	*S25*	*S26*	*S27*	*S28*	*S29*	*S30*	*S31*	*S32*	*S33*	*S34*
—	07	30	—	48	55	—	48	53	—	68	68	—	89	95	95	93	94	97	93
—	01	06	—	26	34	—	30	32	—	54	55	—	89	95	95	93	94	97	94
—	00	03	—	09	13	—	11	12	—	34	37	—	s	81	84	84	s	83	87s
—	06	18	—	53	60	—	52	55	—	78	75	—	95	95	95	95	99	99	93
—	08	22	—	56	63	—	54	57	—	79	77	—	95	95	95	95	99	99	93
—	78	78	—	49	51	—	65	68	—	66	65	—	s	76	86	90	s	79	92
—	21	40	—	50	52	—	49	52	—	69	66	—	63	57	54	68	64	53	86
—	06	15	—	20	24	—	27	26	—	44	44	—	68	64	52	72	66	54	85
—	31s	52	—	51s	55	—	59s	58	—	70	65	—	78	76	65	83	77	67	88
—	00	00	—	00	00	—	00	00	—	02	02	—	66	60	47	71	62	50	24
—	10s	23	—	30s	39	—	37	41	—	55	57	—	77	68	57	78	69	60	88
38	53	52	54	45	46	47	50	50	53	60	59	58	51	33	14	49	44	19	77
15	30	22	38	13	15	18	20s	18	26	25s	30	32	36	14	04	33	33	05	70s
93	s	s	93	s	s	92	s	s	92	s	s	89	66	47	21	69	59	23	91
44	s	s	75	s	s	43	s	s	57	s	s	54	55	29	08	55	48	10	85
00	00	00	00	00	00	00	00	00	00	00	00	00	25	09	03	23	24	03	65s

säure oder Schwefelsäure benutzt. Die Form der Salze, die für die handelsübliche Produkte geeignet ist, ist aber für die Chromatographie auf den mit Formamid imprägnierten Papieren nicht geeignet, da sie in diesem Falle teilweise am Startpunkt bleiben und teilweise gezogene Flecken bilden. Um das Verfahren am einfachsten zu halten, ist es vorteilhaft das Freimachen der Basen direkt auf dem Papier durchzuführen, welches auf verschiedene Art und Weise möglich ist. Man kann gemeinsam mit dem chromatographiertem Stoff auf den Startpunkt einen anderen Stoff auftragen, der die Basen freimacht. Zu diesem Zweck kann man von geeigneter Menge von $NaHCO_3$- oder Na_2SO_3-Lösung Gebrauch machen. Trotzdem diese Methode einfach ist, hat sie doch einen Nachteil, und zwar dass der auf den Startpunkt aufgetragene Stoff einige der Sichtbarmachungsmethoden im Gebiet des Startpunktes stört, sodass die Stoffe, die eventuell in der Nähe des Startpunktes bleiben, der Sichtbarmachung entkommen können. Gleichfalls die Flecken des *p*-Phenylendiamins und dessen Derivate, soweit sie in Form der Salze aufgetragen werden, sind in diesem Falle langgezogen.

Man kann auch einen alkalischen Stoff in die bewegliche Phase zugeben. Zu diesem Zweck ist Pyridin geeignet. Auf diese Weise kann man fast alle geprüften Stoffe chromatographieren; *p*-Hydroxyphenylaminoessigsäure und 2,4-Diaminophenol bleiben auf dem Startpunkt oder in dessen Nähe, *p*-Phenylendiamin und seine Derivate, soweit sie in Form der Salze aufgetragen werden, bilden gezogene Flecken. Um auch bei *p*-Phenylendiamin und seinen Derivaten runde Flecken zu erzielen, können wir anstatt Pyridin Ammoniak benützen. In diesem Falle bilden *o*-Phenylendiamin, *p*-Phenylendiamin, N,N-Diäthyl-*p*-phenylendiamin und N-Äthyl-N-oxyäthyl-*p*-phenylendiamin runde Flecken mit vorteilhaften R_F-Werten; andere Stoffen dieser Stoffgruppe unterliegen dabei teilweiser oder vollständiger Zersetzung.

Die geprüften Substanzen können wir in drei Gruppen, und zwar in Hydroxy-,

TABELLE IV

FÄRBUNG BEI DER SICHTBARMACHUNG

Nr.*	A**	B1	B2	C1	C2	D1	D2	E	F1
1	b	ca	b	d	di	ma	oa, nachträglich ml	nb	sa, nachträglich a
2	b	sra, mbf	a mit w Rand	d	ai	w	l	nachträglich sb	sa, nachträchlich al
3	b	ac	ai	na	ai	a	a, nachträglich la	di	ra, nachträglich a
4	b	sa, mbf	ma mit w Rand	d	ai	sma	l	nachträglich sb	sa, nachträglich la
5	b	sa, mbf	ma mit w Rand	d	ai	sma	l	nachträglich sb	sa, nachträglich la
6	b	sac, mbf	w mit rl Rand	d	ai	oa, bald verschwindet	se, nachträglich w	ma, nachträglich l bis d	o, nachträglich ma bis sd
7	b	a	l	zg	ai	a, mgf Rand	sma	l, nachträglich a	sda
8	b	zc	bl	d	ai	al mit mb Rand	gb	sl, nachträglich d	l, nachträglich la
9	b	ac	a mit zg Rand	d	ai	nachträglich l	nachträglich l	l, nachträglich db	la, nachträglich al
10	b	zc	a mit l Rand	d	ad	sl	gb	ssl	sl
11	b	z ra	gb mit a Rand	ia	ia	k, in SL al	l	al, nachträglich d	lr, nachträglich al
12	a, nachträglich ba	a	l	nachträglich a, cf	nachträglich da	ca bis bg, cf, in SL o bis ra	sma	nachträglich oa	la, nachträglich sda bis d
13	bg, nachträglich b	z cg	rl mit m Mitte, mbf	di	d	bg bis l, in SL sl	lr, nachträglich bl	bg, nachträglich db	bg bis ibl, in SL ss
14	pr, nachträglich b	e	w, mbf	pr, nachträglich i	pr, nachträglich d	e bis dl, in SL sl	b bis l	er, nachträglich sdl	mer bis bl oder rl, in SL ss
15	pr, nachträglich b	e	w, mbf	pr, nachträglich i	pr, nachträglich d	lr bis dl, in SL sl	bl, nachträglich l	pr, nachträglich sdl	ner bis rl oder bl, in SL ss
16	b	in SL ca	ib in SL	na, in SL ss	in SL da	in SL r	in SL ra	in SL r	lr

Nr.*	F2**	G	H	I	J1	J2	J3	K1	K2
1	sa, nachträglich la	ss ca	w, nachträglich ac	nachträglich mac	sa	sa	mac	sna	na
2	a, nachträglich la	ssa	w, nachträglich sa Rand	w	sac	sac	ma	a	i
3	a	sma, in SL mla	nachträglich a	ma	c	c	mca	sca	sa
4	la, nachträglich a	nachträglich sca	w, nachträglich sla	w	sac	sag	sga	ssa	i
5	la, nachträglich a	nachträglich sca	w, nachträglich sla	w	sac	sag	sga	ssa	i
6	ssca, in SL sbg	ssca, in SL sbg	mo, bald verschwindet	w	ssa	ssa	mca	sac	—
7	ssa	c bis ma, scgf	ca	ac	sc	sc	sag bis a	or	r
8	ib, nachträglich al	ac bis mb, cgf	nachträglich al	nachträglich l	ssa	ssa	ga	in SL r	in SL ar, nachträglich la
9	sa	sc bis rba, s cgf, in SL gc	nachträglich sa	nachträglich bl Rand	sac	sac	sag bis gb	in SL sdb	in SL ig
10	sa	c, scgf	m	—	sc	sc	a	sa	ra
11	sda, nachträglich la	sc bis ar, s cgf, in SL ma	or, nachträglich rl	e, nachträglich rl	a bis ra	a bis ra	ra bis a	sa	sna
12	la, nachträglich al	ac, cbf, in SL ar	ac, mef	ac bis zl, cf	ssa	sar	sar bis a	ssa	sa
13	ra bis bl, in SL ss	mr bis nr, mrf	bd, nachträglich l	nachträglich bg bis bl	ssa	lr bis lb	lr bis lb	ra bis lr	ra bis il
14	sl bis bl, in SL ss	c bis ar, cgf, in SL c bis lr	er, nachträglich lb	zr, nachträglich bl	c	c bis lr	a bis lr	ar bis rl	r
15	sl, bis bl, in SL ss	ac bis ar, in SL ac bis kr	pr, nachträglich lb	zr, nachträglich bl	c	c bis lr	a bis lr	ar bis rl	r
16	lr	c bis a, mgf, in SL c bis a, mraf	nachträglich sal	nachträglich sal	sac	ar	r	ib	nl

* Siehe Tabelle I.
** A–K2 siehe experimenteller Teil. a = braun; b = blau; c = gelb; d = grau; e = rosa; f = Fluorescenz in U.V. Licht; g = grün; i = schwarz; k = karminrot; l = violett; m = hell; n = dunkel; o = orange; p = purpurrot; r = rot; s = schwach; ss = sehr schwach; w = weiss; z = schmutzig; SL = saures Lösungsmittel.

Aminohydroxy- und Diaminoverbindungen, einteilen. Bei der Erhöhung der Polarität der beweglichen Phase (eluotrope Reihe Hexan–Benzol–Chloroform–Äthylacetat[9]) sollten sich auch die R_F-Werte der einzelnen Stoffe erhöhen; dies ist nur für Aminohydroxyverbindungen völlig gültig. Bei den Hydroxyverbindungen verursacht Chloroformzugabe zu Benzol in Anwesenheit von Pyridin nur eine sehr kleine Erhöhung, in einigen Fällen eher kleine Erniedrigung der R_F-Werte. Bei den Diaminoverbindungen verursacht die Zugabe des Äthylacetats zu Chloroform praktisch in allen Fällen eine Erniedrigung der R_F-Werte, während bei weiterer Zugabe des Äthylacetats die R_F-Werte wieder höher werden. Dieses verschiedenartige Verhalten der Stoffe unterstützt günstig die Möglichkeit der einwandfreien Trennung aller geprüften Stoffe (Tabelle III).

Für die Trennung der Diaminostoffe kann man sich auch stark saurer Systeme bedienen. In diesen Systemen haben die meisten geprüften Stoffe und zwar besonders die Polyhydroxyverbindungen hohe R_F-Werte. Die R_F-Werte der Aminohydroxyverbindungen sind einigermassen niedriger, aber die relativ kleinen Unterschiede in ihren R_F-Werten erniedrigen die Bedeutung dieser Systeme für ihre Trennung. Vorteilhaft sind diese Systeme für die Trennung des *o*- und *p*-Phenylendiamins, N,N-Diäthyl-*p*-phenylendiamins, N-Äthyl-N-oxyäthyl-*p*-phenylendiamins und 2,4-Diaminophenols. Auch *p*-Hydroxyphenylaminoessigsäure kann man in diesem System chromatographieren, aber ihr R_F-Wert ist fast derselbe, wie des *p*-Aminophenols. Allgemein werden die R_F-Werte in diesen Systemen mit erhöhtem Anteil des niedrigeren Alkohols in beweglicher Phase erniedrigt. Wenn man in der beweglichen Phase eine schwächere Säure, z.B. Essigsäure, benützt, trennen sich die Substanzen nur schlecht; in den meisten Fällen bilden sich da gezogene Flecken, besonders in den Fällen wo die Stoffe in Form der Salze mit starken anorganischen Säuren aufgetragen wurden[10].

Für die Identifizierung der *p*-Hydroxyphenylaminoessigsäure sind weder Systeme mit Formamid, noch saure Systeme geeignet. Man kann das Papier mit Kaliumchlorid imprägnieren und als bewegliche Phase eine Mischung von *n*-Butanol–Äthanol–Kaliumchlorid benützen. *p*-Hydroxyphenylaminoessigsäure wird dabei deutlich von den anderen Stoffen dieser Gruppe, die höhere R_F-Werte unter eventueller Zersetzung der Stoffe haben, abgetrennt.

Die Sichtbarmachung kann auf verschiedene Weisen durchgeführt werden[9]. Als universale Methode kann man das Besprühen mit einem frisch bereiteten Gemisch von Kaliumhexacyanoferrat(III)-lösung und Eisen(III)-chloridlösung benützen, wobei blaue, in einigen Fällen braunstichige Flecken entstehen. Andere Sichtbarmachungsmethoden, die in Tabelle IV angeführt sind, dienen uns zu spezieller Unterscheidung einzelner Stoffe auf Grund verschiedener Färbungen. In diesen Fällen entsteht schon manchmal eine gewisse Färbungsänderung, je nach dem System, in dem die Chromatographie durchgeführt wurde.

ZUSAMMENFASSUNG

Es wurde eine Methode zur Trennung und Identifizierung der 16 Stoffe, die als Entwicklersubstanzen in verschiedenen Handelsprodukten der Entwickler benützt werden, ausgearbeitet. Die Stoffe werden in neutralen oder basischen Systemen auf mit Formamid imprägnierten Papieren, oder in sauren Systemen auf unvorbehandel-

ten Papieren und in einem neutralen System auf mit Kaliumchlorid imprägniertem Papier, getrennt.

SUMMARY

A method has been developed for the separation and identification of 16 substances that are used as developing agents in various commercial photographic developers. These substances are separated by chromatography on paper impregnated with formamide using neutral or basic solvent systems, or on untreated paper using acid systems, or on paper impregnated with potassium chloride using a neutral solvent.

LITERATUR

1 J. Horyna, J. Bulušek und J. Borecký, *Tschechoslow. Pat.*, 105,348 (1962).
2 J. H. Panell und J. E. Louvale, *Anal. Chem.*, 25 (1953) 1566.
3 B. Mariani und P. Martinelli, *Sci. Ind. Phot.*, 27 (1956) 1.
4 K. Futaki, *Pharm. Bull. (Tokyo)*, 4 (1956) 453.
5 J. Šimek, *Chem. Prumysl*, 10 (1960) 403.
6 A. Eckert und R. Endler, *J. Prakt. Chem.*, 104 (1922) 81.
7 E. Sarauw, *Ann.*, 209 (1881) 105.
8 P. Heinrich und W. Schuler, *Helv. Chim. Acta*, 30 (1947) 886.
9 I. Hais und K. Macek, *Handbuch der Papierchromatographie*, VEB Gustav Fischer Verlag, Jena, 1958.
10 J. Gasparič und M. Večeřa, *Mikrochim. Acta*, (1958) 68.

PAPER CHROMATOGRAPHY OF FLAVIN ANALOGUES*

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INTRODUCTION

One of the most commonly used and sensitive means for purification and detection of flavins is paper chromatography. HUENNEKENS AND FELTON note that as little as 0.01 μg of flavin can be detected by this technique when the paper chromatograms are viewed under ultraviolet light[1]. In his review on flavin coenzymes[2], BEINERT points out some of the chromatographic methods which have evolved in the study of flavins. Many recent investigations have used paper chromatography in the purification and estimation of riboflavin and its biological derivatives. The paper chromatographic separation of riboflavin from its natural coenzyme forms, flavin mononucleotide and flavin adenine dinucleotide, has been investigated by CRAMMER[3], YAGI[4], and more recently by WHITE AND LINCOLN[5] and by TRAVIS AND ROBINSON[6]. Similar solvents and resolution techniques have been used by WHITBY[7] and by HUENNEKENS *et al.*[8] for the detection of heretofore unknown flavin compounds in biological materials. The paper partition chromatography of riboflavin decomposition products was investigated by HAIS AND PECAKOVA[9].

The synthesis of flavin analogues for ascertaining exact relationships of structure to biological activity is continuing as an active area of research. FORTER AND KARRER studied the behavior of some twenty synthetic flavins upon paper chromatography[10]. However, recent advances in the syntheses of new flavins has increased considerably both the total number of such analogues and the availability of flavins bearing particular substituents which permit more extensive delineation of the contribution of structure to physico-chemical behavior, especially as regards mobility with solvents on paper chromatograms. A need for such extension of information on the chromatographic properties of flavin analogues has stemmed from studies of the flavin specificities of enzymes, particularly flavokinase[11-14]. The present paper represents a more comprehensive compilation of data on the paper chromatography of flavins and the correlation of structure to R_F values.

EXPERIMENTAL

Procedure

Spots of 5 μl volume containing 2 μg of flavin were applied in a darkened room**

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** Considerable care must be taken to avoid light when working with flavin solutions which are readily photodecomposed, especially in alkaline solvents.

at 2 cm intervals along a line 5 cm from the bottom of sheets of Whatman No. 1 paper, usually 40 cm in height and 50 cm in width. The air-dried sheets were stapled into cylinders and placed upright in glass chromatogram jars, usually 45 cm in height and 25 cm in width, which contained 300 ml of solvent previously equilibrated with the internal atmosphere. The jars were kept in the dark at 25° while solvent was allowed to ascend to within approximately 5 cm of the top of the paper. The chromatograms were removed, the solvent front marked, and the paper dried in a current of air generated in a Reco oven. The flavin spots were marked while their location was observed under ultraviolet light and, whenever possible, by observation under incandescent light as well. Most such flavin compounds exhibit a yellow to orange color in white light and a more intense, yellowish green fluorescence under an ultraviolet lamp.

All flavins were run as duplicates at least twice in the solvents used. Suitable standards, *e.g.* riboflavin, also were included on each chromatogram.

Solvents

The three solvents chosen for their differing pH and polarity are those which, as reported by HUENNEKENS AND FELTON[1], have been found repeatedly satisfactory in the resolution of flavins and their naturally occurring forms:

S_1 = 5 % aqueous Na_2HPO_4
S_2 = *n*-butyl alcohol–acetic acid–water (4:1:5, upper phase)
S_3 = phenol–*n*-butyl alcohol–water (160:30:100, lower phase).

The alkaline solvent, S_1, was used by CARTER[15] in a study of the paper chromatography of purine and pyrimidine nucleotides and has been utilized in the chromatography of flavins and their nucleotides by DIMANT *et al.*[16] and by TRAVIS AND ROBINSON[6]. The acid, more organic S_2 has been used for flavin chromatography by several workers including HAIS AND PECAKOVA[9], WHITBY[7], YAGI[4], and DIMANT *et al.*[16]. Solvent S_3, also used by HUENNEKENS and his coworkers[1,8,16], generally permits the greatest migration of non-phosphorylated flavins.

RESULTS AND DISCUSSION

The correlation of R_F values with structure of a flavin may be considered in terms of substituent functions in the different positions of the parent molecule, riboflavin:

```
        OH OH OH
    CH2-C--C--C-CH2OH
    |   H  H  H
    | 1' 2' 3' 4' 5'
    N     N
CH3-[7 8] 9 [1 2]=O
CH3-[6 5] 10 [4 3]NH
    N     C
          ||
          O
```

Substitution in the side chain

The effects of side chain substitution at position 9 of the alloxazine ring on R_F values of flavins can be seen in Table I. The R_F values obtained for 6,7-dimethylalloxazine (lumichrome), 6,7,9-trimethylisoalloxazine (lumiflavin), and 6,7-dimethyl-9-(1'-D-ribityl)-isoalloxazine (D-riboflavin) are in good agreement with those reported by

TABLE I

EFFECTS OF SUBSTITUTION IN THE SIDE CHAIN (POSITION 9)

Compound	R_F *in solvent system**		
	S_1	S_2	S_3
6,7-Dimethyl substituted			
Alloxazine	0.05	0.62	0.88
9-Methylisoalloxazine	0.15	0.40	0.95
9-Phenylisoalloxazine	0.11	0.68	0.87
9-Formylmethylisoalloxazine	0.15	0.40	0.88
9-(β-Hydroxyethyl)-isoalloxazine	0.26	0.48	0.92
9-(1′-D-Ribityl)-isoalloxazine	0.30	0.28	0.78
9-(1′-D-Arabityl)-isoalloxazine	0.36	0.29	0.80
9-(1′-L-Arabityl)-isoalloxazine	0.37	0.29	0.80
9-(1′-D-Lyxityl)-isoalloxazine	0.35	0.34	0.78
9-(1′-L-Lyxityl)-isoalloxazine	0.33	0.30	0.79
9-(1′-L-2-Deoxylyxityl)-isoalloxazine	0.32	0.47	0.91
9-(1′-D-Sorbityl)-isoalloxazine	0.36	0.24	0.75
9-(1′-D-Dulcityl)-isoalloxazine	0.38	0.21	0.73
9-(1′-D-Rhamnityl)-isoalloxazine	0.36	0.40	0.87
6,7-Dichloro substituted			
9-(1′-D-Ribityl)-isoalloxazine	0.28	0.45	0.75
9-(1′-D-Arabityl)-isoalloxazine	0.36	0.48	0.77
9-(1′-L-Lyxityl)-isoalloxazine	0.32	0.43	0.72
9-(1′-D-Xylityl)-isoalloxazine	0.32	0.41	0.75
9-(1′-D-Sorbityl)-isoalloxazine	0.34	0.36	0.67
9-(1′-D-Dulcityl)-isoalloxazine	0.39	0.36	0.70
9-(1′-D-Mannityl)-isoalloxazine	0.37	0.43	0.70

* Solvent systems: S_1 = 5% aqueous Na_2HPO_4; S_2 = *n*-butyl alcohol–acetic acid–water (4:1:5, upper phase); S_3 = phenol–*n*-butyl alcohol–water (160:30:100, lower phase).

HUENNEKENS AND FELTON[1]. As shown by FORTER AND KARRER[10] for some of the flavins listed in Table I, increasing the number of hydroxyl groups on the side chain causes an increase of the R_F values in a more aqueous solvent, *e.g.* 5% Na_2HPO_4. This may be seen in the proportionally higher values found in the series where position 9 bears no substituent, a methyl group, a β-hydroxyethyl group, a pentityl chain, a 5′-methylpentityl chain, and a hexityl chain, respectively. It is interesting to note, however, that the analogue with a methyl group in this position moves more rapidly in aqueous Na_2HPO_4 and phenol–butanol–water, but less rapidly in butanol–acetic acid–water, than does the unsubstituted 6,7-dimethylalloxazine. This observation is in line with the generally greater solubility of the former flavin, but emphasizes the fallacy of an unqualified assumption that an increase in the number of alkyl groups on the flavin structure elicits a decrease in R_F values in aqueous solvents and an increase in organic solvents[10]. Substitution of position 9 with a relatively hydrophobic phenyl group results in an analogue which has a low R_F value in aqueous Na_2HPO_4, but which value is higher than lumichrome. Less difference in R_F values is seen for longer polyhydroxy chain lengths, *e.g.* L-arabityl *versus* D-dulcityl, where the relative effect of one more alcoholic function is less than at shorter chain lengths. Changes in configuration of a hydroxyl group about only one epimeric center result in minor changes in mobility in the three solvents. The R_F values for D- and L- forms of a

glycityl chain, *e.g.* D-arabityl and L-arabityl, are not significantly different. As expected, a similarity in relative mobilities of those of the above dimethylglycitylisoalloxazines tested previously in a butanol–formic acid–water system[10] was found with the butanol–acetic acid–water solvent used here. The series of pentityl and hexityl flavins with chloro substituents in positions 6 and 7 of the ring have R_F values which are close to but significantly different from their corresponding dimethyl analogues. The dichloro flavins are slightly less water soluble.

Terminal phosphorylation in the side chain

The effects of an orthophosphate ester at the terminal 5′-hydroxymethyl group of the side chain can be seen in Table II. The gross effect of the highly polar phosphoric acid residue is to increase sizably the mobilities of flavins in aqueous solvents, especially in the alkaline Na_2HPO_4 system where effective ionization and salt formation of the flavin phosphate occurs. Moreover, a decrease is found in partially organic

TABLE II

EFFECTS OF TERMINAL PHOSPHORYLATION IN THE SIDE CHAIN (POSITION 5′)

Compound	R_F *in solvent system**		
	S_1	S_2	S_3
5′-Monophosphate substituted isoalloxazines			
6,7-Dimethyl-9-(1′-D-ribityl)-	0.56	0.06	0.09
6,7-Dimethyl-9-(1′-D-arabityl)-	0.61	0.09	—
6,7-Dichloro-9-(1′-D-ribityl)-	0.54	0.12	—
6-Methyl-9-(1′-D-ribityl)-	0.64	0.06	—

* Solvent systems used are described under Table I.

solvents, especially in acid conditions where ionization of the phosphate ester is suppressed. These variations in mobility of riboflavin 5′-monophosphate with polarity of solvent have been demonstrated previously[2]. As with the non-phosphorylated D-arabityl compared to D-ribityl flavin, the 5′-monophosphate of the former has slightly higher R_F values than riboflavin 5′-monophosphate, both in aqueous Na_2HPO_4 and in butanol–acetic acid–water. The 5′-monophosphate of 6,7-dichloro-9-(1′-D-ribityl)-isoalloxazine has a slightly lower R_F value in the Na_2HPO_4 but higher in the butanol–acetic acid system than does riboflavin-5′-monophosphate; again this parallels the comparative values for the free flavins.

Substitution in the benzenoid ring

The effects of substitutions in positions 5, 6, and 7 of the benzenoid portion of the isoalloxazine structure can be seen in Table III. The significant variations in R_F values obtained by differential substitution in the aromatic ring portion of flavins is explainable in terms primarily of the differing solubility properties of the substituents *per se* and secondarily to the effects which such functional groups have directly on the electromeric structure and, hence, indirectly on the polarizability of groups in the alloxanoid portion. The solubility conferred by particular substituents in the benzene ring mainly relates directly to the expected solvophilicity of these groups. Their

TABLE III

EFFECTS OF SUBSTITUTION IN THE BENZENOID RING (POSITIONS 5, 6 AND 7)

Compound	R_F in solvent system*		
	S_1	S_2	S_3
Unsubstituted isoalloxazines			
9-(1'-D-Ribityl)-	0.46	0.17	0.75
9-(1'-D-Sorbityl)-	0.53	0.17	0.67
6-Substituted isoalloxazines			
6-Methyl-9-(1'-D-ribityl)-	0.38	0.21	0.76
6-Methoxy-9-(1'-D-ribityl)-	0.30	0.20	0.75
6-Carboxy-9-(1'-D-ribityl)-	0.17	0.20	0.30
6-Ethyl-9-methyl-	0.12	0.74	0.90
6-Chloro-9-methyl-	0.07	0.50	0.53
7-Substituted isoalloxazines			
7-Methyl-9-(1'-D-dulcityl)-	0.49	0.19	0.71
7-Methyl-9-(1'-D-mannityl)-	0.46	0.21	0.73
5,6-Disubstituted isoalloxazine			
5,6-Dimethyl-9-(1'-D-ribityl)-	0.24	0.28	0.78
6,7-Disubstituted isoalloxazines			
6,7-Dimethyl-9-(1'-D-ribityl)-	0.30	0.28	0.78
6,7-Dichloro-9-(1'-D-ribityl)-	0.28	0.45	0.75
6,7-Dibromo-9-(1'-D-ribityl)-	0.16	0.50	—
6-Methyl-7-fluoro-9-(1'-D-ribityl)-	0.29	0.36	0.77
6:7-Benzo-9-(1'-D-ribityl)-	0.11	0.91	—
6,7-Dimethoxy-9-methyl-	—	0.40	0.95
6:7-Tetramethylene-9-methyl-	0.12	0.54	0.91

* Solvent systems used are described under Table I.

positions in the benzene ring appear to be relatively unimportant. For example, whether the methyl substituents are in both positions 6 and 7, as in riboflavin, or in both positions 5 and 6, as in isoriboflavin, have negligible effects on the comparative migrations of these flavins in all three solvents employed. Also, the relative mobilities of the 6-methyl and 6,7-dimethyl derivatives of 9-(1'-D-ribityl)-isoalloxazine are comparable to the relative mobilities of the 7-methyl and 6,7-dimethyl-flavins which bear a dulcityl side chain (*cf.* Table I). Therefore, presence of methyl groups in positions 5, 6 and 7 are approximately equivalent with respect to solubility in the chromatographic solvents. In a comparable series of symmetrical 6,7-disubstituted flavins, *e.g.* dimethyl, dichloro, and dibromo analogues of 9-(1'-D-ribityl)-isoalloxazines, the R_F values are a reflection of the solubilities of these flavins in the solvents. Lack of substitution or substitution with different groups in either position 6 or in both 6 and 7 positions produces flavins which have the expected differences in R_F values.

Substitution in the alloxanoid ring

The effects of substitution in positions 2 and 4 of the alloxanoid portion of the isoalloxazine structure can be seen in Table IV. No great effect of substituents in po-

TABLE IV

EFFECTS OF SUBSTITUTION IN THE ALLOXANOID RING (POSITIONS 2 AND 4)

Compound	R_F *in solvent system**		
	S_1	S_2	S_3
L-(D-Ribityl)-2,3-diketo-6,7-dimethyl-1,2,3,4-tetrahydroxyquinoxaline	0.42	0.47	0.73
2-Substituted-6,7-dimethylisoalloxazines			
2-Thio-9-(1'-D-ribityl)-	0.30	0.30	0.96
2-Imino-9-(1'-D-ribityl)-	0.20	0.30	0.78
2-Benzylazino-9-(1'-D-ribityl)-	0.28	0.19	0.96
2-Phenylamino-9-(1'-D-ribityl)-	0.30	0.30	0.92
2-(β-Hydroxyethylamino)-9-(1'-D-ribityl)-	0.28	0.34	0.85
2-Morpholino-9-(1'-D-ribityl)-	0.21	0.41	0.94
2-Methylmercapto-9-(1'-D-ribityl)-	0.29	0.31	0.94
2-Substituted-6,7-dimethoxyisoalloxazine			
2-Thio-9-methyl-	—	0.41	0.96
4-Substituted-6,7-dimethylisoalloxazine			
4-Imino-9-(1'-D-ribityl)-	0.10	0.50	0.88

* Solvent systems used are described under Table I.

sition 2 can be seen from R_F values obtained using aqueous Na_2HPO_4; greater differences are apparent in the partially organic, acid solvents. The polarization effects in position 2 of these analogues are relatively similar, whereas their solubilities in organic solvents are quite dissimilar. Even under the apparently mild conditions of chromatography used, the alkaline pH of Na_2HPO_4 permits nucleophilic displacement of the sulfur in the 2-thio analogue to form riboflavin. This effect has been noted before[14,17]. The resultant continuous production of riboflavin gives rise to a less defined spot on paper chromatograms where the 2-thio and 2-oxy compounds overlap. Suitable stability and separation of both these flavins is found with phenol–butanol–water as solvent. Replacement of the 4-carbonyl of riboflavin with a 4-imino function markedly alters the mobility of this flavin in all three solvents. This finding may be explained by the altered tautomerizable nature of the latter compound about positions 3 and 4. The 4-imino flavin has a decreased ability to form an enamino tautomer analogous to the polar, ionized enol structure of riboflavin induced by an electrophilic agent. Studies of metal chelation by flavins[18] and, more recently, the binding of this flavin to flavokinase[14] substantiate this finding.

SUMMARY

A comprehensive paper chromatographic study has been made of the mobilities of some fifty flavin analogues in three solvent systems which are different with respect to pH and polarity. Specific alterations in the structures of flavins influence the solubility characteristics and have a direct and generally predictable effect on R_F values:

1. Increasing the number of hydroxyl groups on the side chain increases the solubility of flavins in aqueous solvents. Relatively smaller differences are seen among longer chains than shorter ones.

2. Phosphorylation of the hydroxymethyl group terminal to the side chain greatly increases the mobility of flavins in alkaline, aqueous solvents and decreases their mobility in acid, organic solvents.

3. The solubility and resultant mobility conferred by particular substituents in the benzenoid portion of the isoalloxazine ring mainly relate to the known solubility of the substituent group. Their positions in the benzene ring of flavins appear to be relatively unimportant.

4. Effects of substitution in position 2 of the alloxanoid portion of the isoalloxazine ring appear similar in an alkaline, aqueous solvent, but dissimilar in acid, organic solvents. A 4-imino function markedly alters the mobilities in all three solvents.

REFERENCES

[1] F. M. Huennekens and S. P. Felton, in S. P. Colowick and N. A. Kaplan (Editors), *Methods in Enzymology*, Vol. III, Academic Press, Inc., New York, 1957, p. 957.
[2] H. Beinert, in P. D. Boyer, H. Lardy and K. Myrback (Editors), *The Enzymes*, Vol. II, Academic Press, Inc., New York, 1960, p. 385.
[3] J. L. Crammer, *Nature*, 161 (1948) 349.
[4] K. Yagi, *Med. Biol. (Tokyo)*, 25 (1952) 266.
[5] I. G. White and G. J. Lincoln, *Biochem. J.*, 76 (1960) 301.
[6] J. Travis and A. D. Robinson, *Can. J. Biochem. Physiol.*, 40 (1962) 1251.
[7] L. G. Whitby, *Nature*, 166 (1950) 479.
[8] F. M. Huennekens, D. R. Sanadi, E. Dimant and A. I. Schepartz, *J. Am. Chem. Soc.*, 75 (1953) 3611.
[9] I. M. Hais and L. Pecakova, *Nature*, 163 (1949) 768.
[10] W. Forter and P. Karrer, *Helv. Chim. Acta*, 36 (1953) 1530.
[11] D. B. McCormick, *Federation Proc.*, 21 (1962) 239.
[12] D. B. McCormick, *J. Biol. Chem.*, 237 (1962) 959.
[13] D. B. McCormick and R. C. Butler, *Biochim. Biophys. Acta*, 65 (1962) 326.
[14] D. B. McCormick, C. Arsenis and P. Hemmerich, *J. Biol. Chem.*, in the press.
[15] C. E. Carter, *J. Am. Chem. Soc.*, 72 (1950) 1466.
[16] E. Dimant, D. R. Sanadi and F. M. Huennekens, *J. Am. Chem. Soc.*, 74 (1952) 5440.
[17] F. Muller, P. Hemmerich and H. Erlenmeyer, *Experientia*, 18 (1962) 498.
[18] I. F. Baarda and D. E. Metzler, *Biochim. Biophys. Acta*, 50 (1961) 463.

Notes

Structural effects on quantitative gas-chromatographic detector response

Methyl esters of dicarboxylic acids

The use of gas–liquid chromatographic techniques for quantitative analysis implies that the detector responds uniformly to the various compounds to be assayed. Previous work relevant to this assumption has involved the response of thermal detectors[1–4]. The response of the β-ray argon detector has not been studied extensively, although LOVELOCK[5–7] indicates a trend towards constant sensitivity with increasing molecular weight for several types of organic compounds. It has been predicted that schemes similar to those found for thermal conductivity cells will be necessary if β-ray detectors are to be used for compounds of low molecular weight, with the response leveling off at a constant value on a weight basis at increasing molecular weights[8].

The present study is an investigation of the molar responses of a β-ray argon detector towards the dimethyl esters of the straight chain α,ω-dicarboxylic fatty acids.

Experimental

The gas chromatograph was a Barber-Colman Model 15 with radium ionization detector. The column was 7.5 % diethylene glycol adipate on Gas-chrom P, 100–140 mesh. The column temperature was 130°, that of the flash heater 170–220°, and the temperature of the detector 267°. The detector was operated at 750 V, scale 3. The argon flow rate was 24, or 67 ml/min. The areas under the peaks were independent of flow rate.

The compounds under investigation were introduced into the column in diethyl ether solution, using Hamilton syringes. Dimethyl malate was used in most of the determinations as an internal standard.

Results and discussion

The molar responses, both absolute and relative (dimethyl malate = 1), show an alternation of peak areas with the number of carbon atoms per molecule; this alternation, however, tends to level off at seven carbon atoms (Fig. 1, A to F). Such alternation of properties with the number of carbon atoms has often been observed in the aliphatic straight chain acids; it has been found for melting points, crystal spacings and solubilities[9]. The dicarboxylic acids also show alternations in the antisymmetric COO– stretchings of the sodium and copper salts as well as the magnetic moments of the copper salts[10].

The influence of one carboxyl group upon the other for these properties, among the aliphatic acids and their derivatives, is of course most marked in oxalic acid. It drops off with insertion of methylene groups, being fairly strong in malonic acid

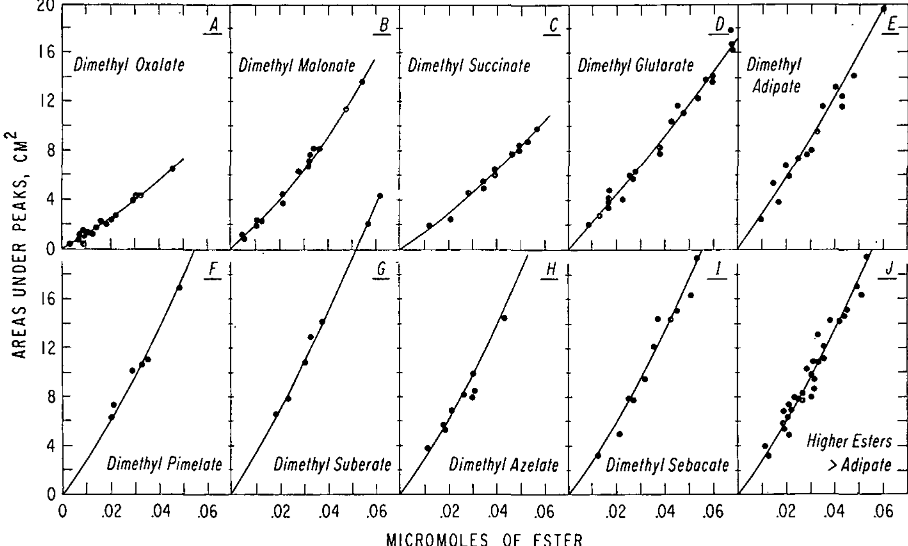

Fig. 1. Detector responses for dimethyl dicarboxylates. Areas under peaks (cm²) as function of μmoles of ester.

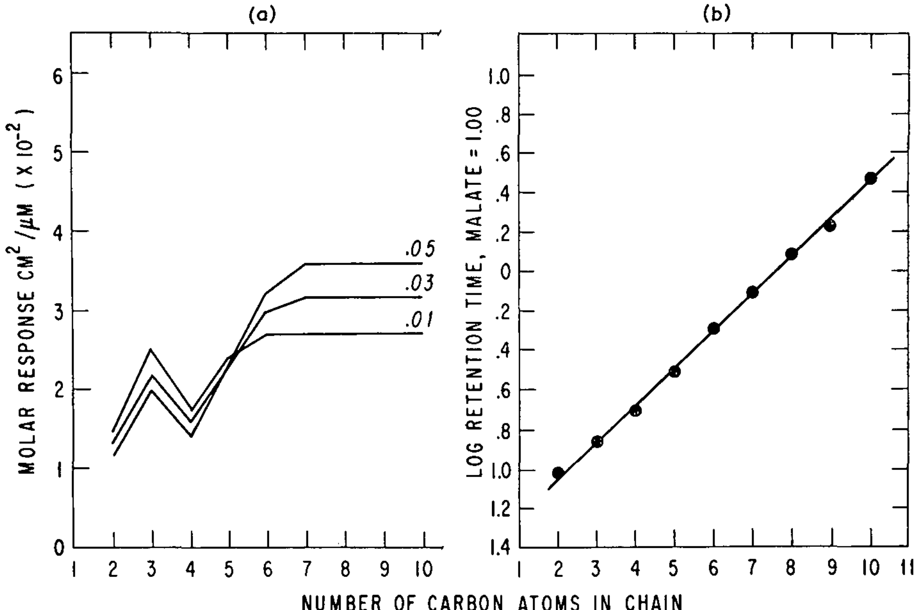

Fig. 2. (a) Alternation of molar responses (cm²/μmole) with number of carbon atoms in chain, at varying amounts of ester, μmoles. (b) Retention times as function of number of carbon atoms in chain; dimethyl malate = 1.00.

(one —CH_2—) and still apparent in succinic acid (two —CH_2—); when the functional groups are separated by three or more methylene groups the interaction disappears, probably owing to mutual repulsion of the two groups, which causes the chain to swing into a position allowing maximum distance between the groups. The succeeding members of the series follow a more smoothly changing course.

These effects are borne out in Figs. 1 and 2a. The difference in slopes in the first four esters (oxalate–glutarate) is readily apparent. For esters higher in the series than dimethyl adipate a common curve fits all within experimental error (Fig. 1J). Due to the curvature of the plots, molar responses increase with increasing amounts of ester, but the alternation in response persists up to the highest levels investigated (Fig. 2a).

As might have been expected, the retention times of this series follows the usual semi-logarithmic curve when plotted as a function of number of carbon atoms (Fig. 2b).

The basic cause of these alternations is probably the ionization potential of the molecule, although data to support such an hypothesis are scanty. FIELD AND FRANKLIN[11] list many ionization potentials of organic compounds, but none of the present series. In general, ionization potentials as function of the number of carbon atoms follow a smooth curve without alternations. In the series of the *n*-alkyl benzenes, the ionization potential is slightly lower for toluene than it is for either benzene or ethylbenzene[11], but this may be due to the direct influence of the benzene ring. Mass spectrometric data are available for a limited number of these methyl esters[12]. If the m/e value of the most intense peak of the pattern is used as criterion, some indications of similar effects are found. For even values of n ($n = 6, 8, 10, 20$ and 24) m/e is relatively low (< 100), while for the single compound listed for which n is odd ($n = 11$), m/e equals 152. Thus it is possible, although by no means really proven, that a relationship exists between responses and ionization potential.

Clearly, this possibility of alternations of response should be kept in mind when quantitation of mixtures by gas–liquid chromatography is attempted.

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1 A. B. LITTLEWOOD, *Nature*, 184 (1960) 1631.
2 D. M. ROSIE AND R. J. GROB, *Anal. Chem.*, 29 (1957) 1263
3 A. E. MESSNER, D. M. ROSIE AND P. A. ARGABRIGHT, *Anal. Chem.*, 31 (1959) 230.
4 G. R. JAMIESON, *J. Chromatog.*, 3 (1960) 464.
5 J. E. LOVELOCK, *J. Chromatog.*, 1 (1958) 35.
6 J. E. LOVELOCK, A. T. JAMES AND E. A. PIPER, *Ann. N. Y. Acad. Sci.*, 72 (1959) 720.
7 J. E. LOVELOCK, *Anal. Chem.*, 33 (1961) 162.
8 I. G. MCWILLIAM, *Rev. Pure Appl. Chem.*, 11 (1961) 33.
9 L. F. FIESER AND M. FIESER, *Organic Chemistry*, 3rd Ed., Reinhold Publishing Co., New York, 1956, pp. 312, 400.
10 Y. KURODA AND K. J. MASAJI, *J. Phys. Chem.*, 64 (1960) 759.
11 F. H. FIELD AND J. L. FRANKLIN, *Electron Impact Phenomena*, Academic Press, New York, 1957, pp. 105 *et seq.*
12 R. RYHAGE, AND E. STENHAGEN, *Arkiv Kemi*, 14 (1959) 497.

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Use of a spinal needle as an injection device for solid substances in gas chromatography

A relatively inexpensive, readily available item, the B-D Yale Luer-Lok No. 462 LNR Spinal Needle (Quincke) (Becton, Dickinson and Company, Rutherford, N. J.) can serve, without alteration, as an injection device for solid substances into a gas chromatograph. Although several methods of introducing solids into gas chromatographs have been reported[1-3] this method for directly injecting up to a 6 mg sample without instrument modification nor disruption of gas flows is free from complexity. The injection of these sample sizes allows for detection with either thermisters or hot wires and also allows for sample collection for further analysis or investigation.

A spinal needle is shown in Fig. 1, item 1. It is made up of two units, a cannula, item 2, which is essentially a syringe needle, and a stilette, item 3, which is essentially a solid steel rod. The stilette fits into the cannula with a snug to sliding fit. Both the cannula and stilette have beveled tips. Although spinal needles with cannula outer diameters ranging from 0.025 to 0.050 in. have been used, a cannula outer diameter of about 0.035 in. and a stilette diameter of about 0.022 in. was found to be about optimum for injection of 3 to 6 mg samples. These cannula and stilette dimensions correspond to a 20 gauge spinal needle.

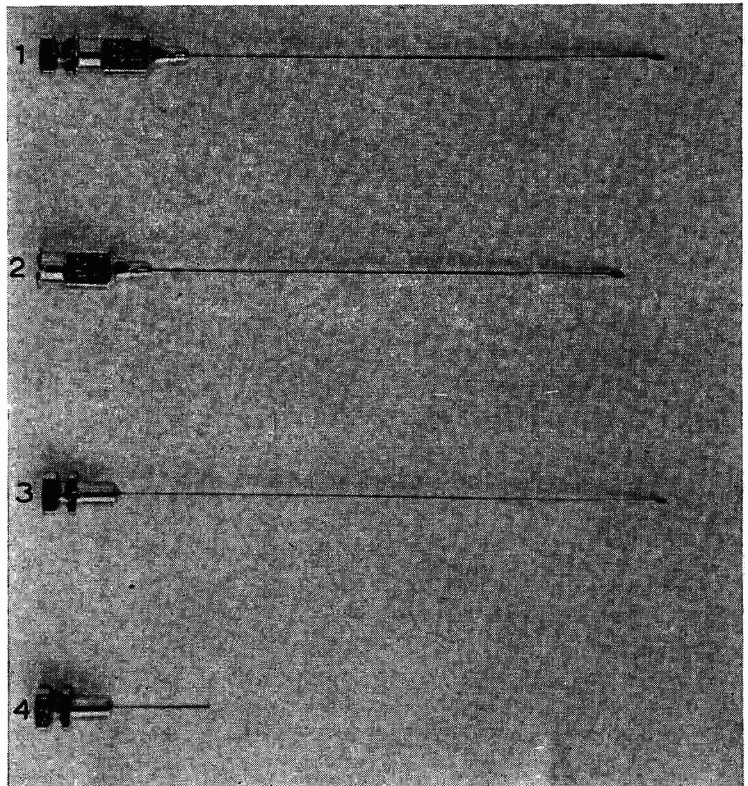

Fig. 1. Injection device (approximately actual size). 1: Spinal needle. 2: Cannula. 3: Stilette. 4: Packing rod.

In order to prevent leakage during use as a solids injector into a gas chromatograph, a little silicone gum rubber liquid phase column packing (F and M Scientific Corp., Avondale, Pa.) may be applied in the vicinity of the plunger end of the stilette and into the hub of the cannula. This, however, is not always necessary.

The device is filled by pulling the stilette into the cannula so as to form a cylindrical cavity at the end of the needle approximately $^1/_4$ to $^1/_2$ in. in depth and forcing the ground sample into this cavity. The ground sample is forced into the cavity by pushing the open end of the needle against a thin, packed layer of ground sample contained in a glass mortar. The packing rod, Fig. 1, item 4, having approximately the same diameter as the stilette but being somewhat shorter in length, is used to aid in the filling of the cavity.

Injection is accomplished in a similar manner as with a liquid or gas syringe, that is, the needle is inserted through an injection port silicone-rubber diaphragm or septum, and the sample is forced out of the cavity of the needle by application of pressure to the stilette. The needle is then immediately withdrawn from the injection port. About a dozen "passes" with a 20 gauge needle are possible before a silicone rubber septum would require replacement.

Quantitative relationships are obtainable by weighing before and after injection; however, "normalization", without sample weighing, is also quite frequently used.

This spinal needle injection technique has been used very successfully by our laboratories for some time where substances with melting points as high as 300° have been successfully chromatographed. This, of course, necessitates a chromatograph capable of providing high temperatures in the injection port and detector block systems. A F and M Model 500 Programmed High Temperature Gas Chromatograph was used for these studies, and the chromatography of these high melting point substances may be the subject of future papers. As an injection device for solid substances, the spinal needle has been found to be quite sturdy and fool-proof. After prolonged usage, the stilette may accumulate a carbon deposit and clogging may occur. The relative low cost of the unit, however, allows for discarding without concern.

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1 R. L. BOWMAN AND A. KARMEN, *Nature*, 182 (1958) 1233.
2 D. B. McCOMAS AND A. GOLDFLEN, *Anal. Chem.*, 35 (1963) 263.
3 A. RENSHAW AND L. A. BIRAN, *J. Chromatog.*, 8 (1962) 343.

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Gefüllte Kapillarsäulen in der Gaschromatographie

Gefüllte Trennsäulen im Durchmesserbereich von 8–2 mm sind in der Gas Chromatographie hinreichend bekannt. Seit MARTIN[1] 1956 die Notwendigkeit kapillarer Säulenquerschnitte für Mikroanalysen beschrieben hat, wurden von GOLAY[2] die Kapillarsäulen, deren Innenwand direkt mit der Trennflüssigkeit imprägniert wird, verwendet. Um hierbei hohe Trennstufenzahlen zu erreichen sind Längen von einigen 10 Metern und mehr erforderlich. Gepackte Glaskapillaren wurden von HALÁSZ UND HEINE[3]) für die Trennung von Äthylen und Butan verwendet. Diese haben allerdings den Nachteil, dass die Herstellung sehr zeitraubend ist.

Wir verwenden seit einiger Zeit gepackte Stahl- bzw. Messingkapillaren mit Durchmessern von 0.5–1 mm. Die Füllung erfolgt mit unbelegtem Trägermaterial (Chromosorb W oder Kieselgur, Körnung 0.1–0.01 mm und 0.09–0.063 mm) mittels eines Vibrators, durch ein auf 2 mm Tiefe konisch erweitertes Ende der Kapillare. Auf diese Weise erzielt man eine gleichmässigere Packung als mit vorher imprägniertem Trägermaterial. Die Füllung wird zweckmässigerweise mit Kapillarstücken von 2–3 m vorgenommen, die sich zum Gebrauch durch Ermetroverschraubungen mit Kapillareinsatz auf beliebige Längen kombinieren lassen. Die Füllung längerer Abschnitte ist jedoch auch möglich.

Die Imprägnierung des Trägers erfolgt nach der Füllung des Rohres am besten auf folgende Weise: an das Reduzierventil einer Stickstoff-Bombe wird ein druckdichter Behälter angeschlossen, der die Trennflüssigkeit in Methylenchlorid gelöst enthält. Mit einem Druck von ca. 2 kg/cm^2 wird die Lösung langsam durch die Kapillare gedrückt. Nach beendeter Imprägnierung erfolgt wie üblich die Trocknung der Säule im Gasstrom und die Alterung unter Temperatureinwirkung.

Die Dosierung der zu analysierenden Proben erfolgt bei Verwendung derartiger Säulen nach dem Splitting-Verfahren über einen Teiler, die Detektion über einen Mikroflammen-Ionisationsdetektor. Die zu dosierende Menge ist bei den gefüllten Kapillarsäulen nicht so kritisch wie bei belegten Kapillaren, da die zur Verfügung

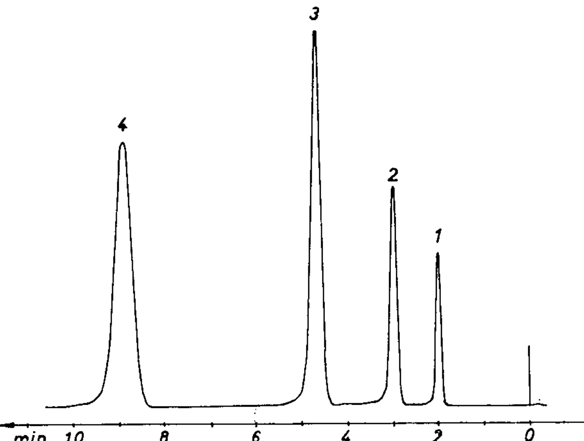

Fig. 1. Gas Chromatogramm von (1) Hexan, (2) Benzol, (3) Toluol, (4) *o*-Xylol. Säule 3 m, I.D. 1 mm, 20 % Dioctylsebacinat auf Chromosorb W, Körnung 0.09–0.06 mm; 125°, 0.65 kg/cm^2 N_2, 20 ml/min; Probenmenge 2 μl, Teilung 1:20; Mikroflammen-Ionisationsdetektor.

stehende Oberfläche, bedingt durch den Träger, grösser ist als bei ungefüllten Kapillaren und daher die Menge der flüssigen Phase erhöht werden kann. Substanzmengen zwischen 0.02 und 0.01 μl werden gut getrennt.

Es wurden für die Trennung von Hexan, Benzol, Toluol und *o*-Xylol gefüllte Kapillarsäulen mit 20% Dioctylsebacinat auf Chromosorb W und für Fettsäuremethylester 20% LAC-728 auf Kieselgur in Längen von 3 m verwendet. Die flüssige Phase wurde zur Imprägnierung in Methylenchlorid gelöst. Die erreichten Trennstufenzahlen lagen zwischen 2600 bis 2800. Die Analysen wurden mit dem Gasofract 300 B durchgeführt (siehe Fig. 1 und 2).

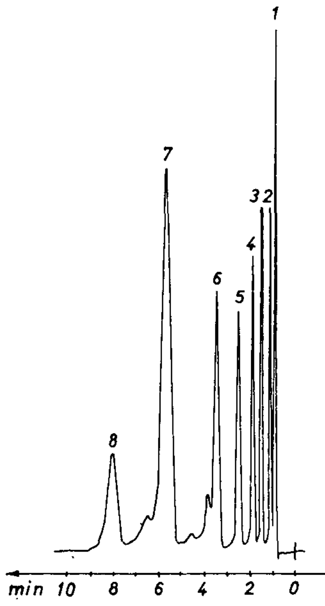

Fig. 2. Gas Chromatogramm von Fettsäuremethylestern in (1) Hexan, (2) Capronsäure, (3) Caprinsäure, (4) Laurinsäure, (5) Myristinsäure, (6) Palmitinsäure, (7) Ölsäure, (8) Linolsäure. Säule 3 m, I.D. 1 mm, 20% LAC-728 auf Kieselgur, Körnung 0.09–0.06 mm; 225°, 1.5 kg/cm^2 N_2, 15 ml/min; Probenmenge 2 μl, Teilung 1:20; Mikroflammen-Ionisationsdetektor.

Es ist ersichtlich, dass man bei gefüllten Kapillarsäulen schon mit erheblich kürzeren Längen, als bei den belegten Kapillaren, auf hohe Trennstufenzahlen kommt. Die Analysenzeit ist kürzer als bei normalen, gefüllten Säulen mit Durchmessern von 6–4 mm. Der Druckabfall bleibt in normalen Grenzen.

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[1] A. J. P. MARTIN, in D. H. DESTY (Herausgeber), *Vapour Phase Chromatography*, Butterworths, London, 1957, p. 2.
[2] M. J. E. GOLAY, in D. H. DESTY (Herausgeber), *Gas Chromatography*, Butterworths, London, 1958, p. 46.
[3] J. HALÁSZ UND E. HEINE, *Nature*, 194 (1962) 971.

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A new colorimetric test for detecting sulfur-containing amino acids

Sulfur-containing amino acids and particularly the S-alkyl cysteines, methionine, and related amino acids are frequently detected colorimetrically by the iodoplatinate procedure[1] or by the iodine azide method[2]. These reagents do not, however, distinguish sulfides from sulfoxides and frequently the latter compounds give weak, indecisive responses. The present study arose from the need for a satisfactory colorimetric reagent for detecting "cycloalliin"[3] (3-carboxy-5-methyl-1,4-thiazane-1-oxide) on paper chromatograms. This amino acid at low concentration gives virtually no color with ninhydrin or with isatin and has only a weak bleaching action on the iodoplatinate reagent.

We have found that when paper chromatograms are sprayed with FEIGL'S[4] reagent for secondary amines (aqueous sodium nitroprusside–acetaldehyde solutions) and then fumed with hydrochloric acid, S-substituted cysteines and methionine yield blue colors and the corresponding sulfoxides give pale violet colors. Both cycloalliin and reduced cycloalliin (3-carboxy-5-methyl-1,4-thiazane) respond to this test. Surprisingly, neither of these compounds gives a blue color before acid treatment, although the original Feigl test is very sensitive for such other cyclic secondary amino acids as proline and pipecolic acid. 3-Carboxy-1,4-thiazane, a homologue (without the methyl group)* of reduced cycloalliin, does yield a blue color with the Feigl test for secondary amines, and also a blue on subsequent treatment with hydrochloric acid, in agreement with our other results. In some cases, N-acylated sulfide or sulfoxide amino acids will respond to the test. As shown in Table I, two N-acetyl derivatives responded, while two N-benzoyl and one carbobenzyloxy derivative gave negative results.

The test is sufficiently sensitive to detect cycloalliin, reduced cycloalliin, S-methyl-L-cysteine and the corresponding sulfoxides on paper chromatograms at levels of 3 γ per spot. Table I lists compounds that give positive responses to the test. Cysteine and cystine yield pale greenish-blue and muddy blue colors, respectively. Those amino acids that do not contain sulfur generally yield muddy green or grey colors which can easily be distinguished from the light blue or lavender of the sulfide or sulfoxide amino acids.

Experimental

Paper. Schleicher and Schüll 589 Blue Ribbon and Whatman No. 1 papers were used**.

Reagents. 2 % w/v sodium bicarbonate in 30 % aqueous ethanol. 2 % w/v aqueous sodium nitroprusside. 20 % v/v acetaldehyde in aqueous solution.

The nitroprusside and acetaldehyde solutions should be kept under refrigeration and mixed in equal proportions before use. The mixed reagent is usually effective for a week when stored in the cold, but best results are obtained with freshly mixed solutions.

Procedure. The developed paper chromatogram, after air-drying, is sprayed with the bicarbonate solution and then dried again. The paper is then sprayed with the mixed acetaldehyde–nitroprusside reagent (proline is a good indicator since it

* Synthesis to be published.

** Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

TABLE I

COLOR REACTION OF SOME S-CONTAINING AMINO ACIDS WITH SODIUM NITROPRUSSIDE–ACETALDEHYDE REAGENT

Amino acid	*Color*
S-Methyl-L-cysteine	Blue
S-Methyl-L-cysteine sulfoxide	Lavender*
S-*n*-Propyl-L-cysteine	Blue
S-*n*-Propyl-L-cysteine sulfoxide	Lavender
S-Allyl-L-cysteine	Blue
S-Allyl-L-cysteine sulfoxide	Lavender
S-(β-Hydroxyethyl)-L-cysteine	Blue
S-(β-Hydroxyethyl)-N-acetyl-L-cysteine methyl ester	Blue
S-(β-Hydroxyethyl)-N-acetyl-L-cysteine methyl ester sulfoxide	Lavender
N-Benzoyl-S-*n*-propyl-L-cysteine sulfoxide	—
Dibenzoyl-mesolanthionine	—
Dicarbobenzyloxy-mesolanthionine	—
Djenkolic acid	Blue
Mesolanthionine	Blue
L-Allocystathionine	Blue
Cycloalliin (sulfoxide)	Violet
Reduced cycloalliin	Blue
3-Carboxy-1,4-thiazane	Blue
Methionine	Blue
Methionine sulfoxide	Blue-violet
Methionine sulfone	Blue-violet

* No significant difference was observed between the *dextro* and *levorotatory* S-methyl-L-cysteine sulfoxides.

invariably gives a strong blue color at this stage), air-dried, and fumed with hydrochloric acid a in suitable chamber for 15–30 sec for sizes of paper up to 24 × 24 cm or 45–60 sec for larger chromatograms. The paper is hung in a fume hood and the colors noted from 15 min to an hour after fuming. The color produced by cycloalliin is stable and often becomes more intense after 24 h, but for most of the compounds tested the color gradually fades after several hours.

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1 H. M. WINEGARD, G. TOENIES AND R. J. BLOCK, *Science*, 108 (1948) 506.
2 E. CHARGAFF, C. LEVINE AND C. GREEN, *J. Biol. Chem.*, 175 (1948) 67.
3 A. I. VIRTANEN AND E. J. MATIKKALA, *Acta Chem. Scand.*, 13 (1959) 623.
4 F. FEIGL, *Spot Tests in Organic Analysis*, 6th Ed., Elsevier, Amsterdam, 1960, p. 273.

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Large divided columns

PILLAY *et al.*[1] have stated well the problems encountered in liquid–solid adsorption column chromatography, and have presented the technique of divided columns for improving the sharpness of separations. To isolate minor constituents from some oils obtained in aroma investigations, hundreds of grams of material may need to be chromatographed. Obviously kilograms of adsorbents must be used, and irregular zone fronts and time of completion of separation become major problems.

A combination of CLAESSON'S recommendation[2] to use a series of columns with diminishing diameters and PILLAY'S technique of dividing the column of adsorbent into shorter sections can be utilized to good effect in large column-work. This combination can be accomplished by assembling the column in sections, which can be varied in size from 8 cm diameter and 40 cm high to 4 cm diameter and 20 cm high. The number of sections used can also be changed according to the amount of adsorbent necessary. The Kontes O-ring connector*, size 40, is a convenient means for connecting the sections (Fig. 1). Grease contamination, as in the case of ball-socket joints, is eliminated with this type of connector, since neoprene or silicone rubber O-rings are used for seals. For tall columns, more tension than that exerted by the conventional No. 65 ball-socket clamp is necessary. The tension needed to stop leakage is obtained by cutting off the hinge and by applying tension at three points with three bolts and nuts (Fig. 2).

The irregular zone front is sharpened as the material passes from one section to another through a zone of solvent above the adsorbent. The "coning" effect is compensated for by the funnel at the bottom of each section, where a plug of glass wool prevents the solid adsorbent from following into the next section. Also, if the column is built in short sections, the weight of the adsorbent does not continue to add and cause the adsorbent to pack so tightly that the resistance to eluant flow becomes great. If 100–200 mesh adsorbent is used, flows of 2 to 4 l per h through 3 to 4 kg of adsorbent in 4 large sections can be easily achieved.

With large quantities and fast separations, the heat of adsorption of strongly adsorbed materials can cause difficulties. But such difficulties can be diminished by proper deactivation of the adsorbent and careful gradation from solvent to solvent.

Since large volumes of eluants are used, a solvent feeding system must be used that provides a constant-head pressure and some insurance against going dry. Fig. 3 shows a simple system which has proved convenient. The lower "S" section from the reservoir to the column provides vertical freedom in assembly. The small reservoir provides a constant-head pressure, and the "goose-neck" curve in the tubing from the reservoir stops any mixing of the liquid as the polarity (therefore, usually the density) of the eluant in the feeders is increased. The two inverted 2 l round bottom flasks with standard-taper joints and offset feeder arms can be used to maintain the level in the reservoir. These feeder flasks can be filled easily by removing the

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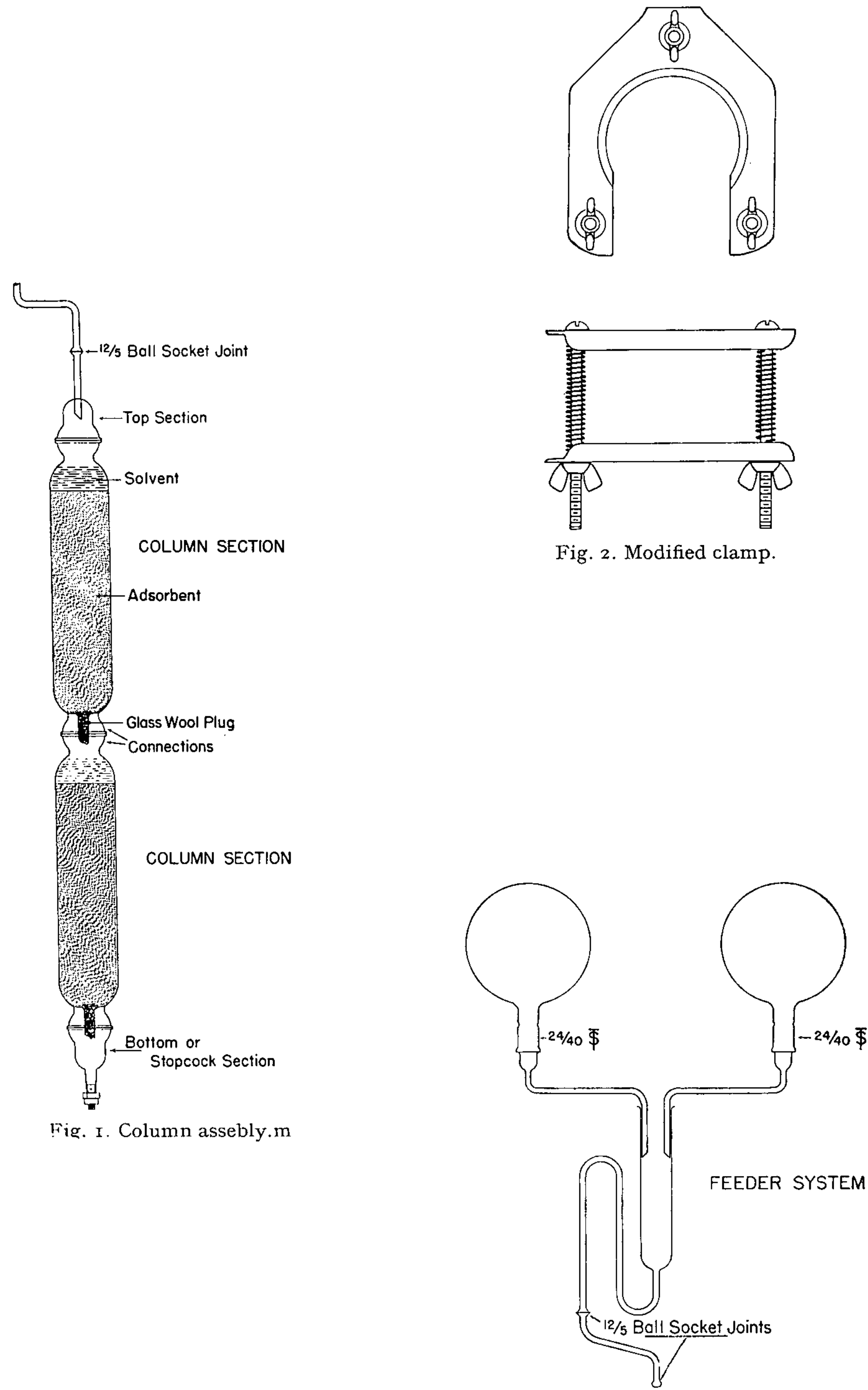

Fig. 1. Column assebly.m

Fig. 2. Modified clamp.

Fig. 3. Solvent feeder assembly.

feeder arms. Since the pressures in the feeder system are small, the ball-socket and standard-taper joints can be assembled without grease.

With the described arrangement, chromatographic separation of several hundred grams of material can be completed in a few days.

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1 P. P. PILLAY, D. S. RAO, C. P. N. NAIR AND E. T. VARKEY, *Chem. Ind. (London)*, (1958) 258.
2 S. CLAESSON, *Arkiv Kemi Mineral. Geol.*, 16 (1957) 24A.

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A spraying method for the preparation of thin-layer chromatoplates*

Thin-layer chromatography on microslides has been found particularly useful for the rapid checking of samples obtained at different stages in the preparation or purification of organic compounds.

In our laboratory the technique has also been used for finding a suitable solvent system for use in column chromatography of steroids. A rapid method has been devised for applying a thin layer of silica gel or alumina on microscope slides. The method involves spraying a slurry of silica gel (10 g in 30 ml of water) with a conventional glass sprayer used for spraying reagents. The process is illustrated in Fig. 1. The precaution

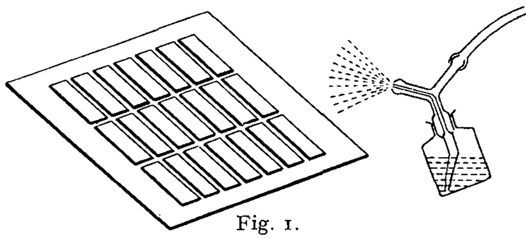

Fig. 1.

should be taken to keep the microslides on a flat surface to secure a uniform coating. The plates can be dried at room temperature or at an elevated temperature (100°, 30 min). The thickness of the coating prepared by this technique ranges around 200 μ–300 μ and 10 g of commercially available Silica Gel G (Merck, Germany) is sufficient to coat 20 microslides (75 × 25 mm).

The technique is quickly learned and has the advantage that the necessary equipment is available in every laboratory.

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* The method was devised by one of us (K.M.) when he worked at the National Institutes of Health, Bethesda 14, Md., U.S.A.

Chromatographie de sucres sur couches minces de silicate de calcium

Le silène EF *, un silicate de calcium hydraté, est un produit industriel très accessible, qui a été proposé pour la chromatographie sur colonne des sucres, particulièrement par la technique d'extrusion[1]. Nous avons constaté que la chromatographie des sucres sur couches minces de silène était également possible, dans de bonnes conditions.

Les plaques (environ 0.2 mm d'épaisseur), sont préparées manuellement et séchées à l'air avant d'être chauffées. La ligne de départ se trouve à 2 cm du bord inférieur. La migration étant relativement lente, il est possible d'accélérer le développement:

(1) en chauffant les plaques 15 h à 110°,

(2) en incorporant au silène de la Célite 535 (lavée à l'acide chlorhydrique concentré) (11 g de silène EF pour 3 g de Célite 535 et 700 mg d'acétate de sodium — ce dernier produit semble améliorer légèrement les séparations).

Un liant, tel que le plâtre, n'est pas nécessaire, car les plaques restent solides, même après 20 h à 110°C.

Deux solvants nous ont donné de bons résultats:

(1) *n*-Butanol 100 cm³, complèter à 114 cm³ avec de l'eau.

(2) *n*-Butanol 92 cm³, complèter d'abord à 100 cm³ avec de l'acétate de butyle, puis à 114 cm³ avec de l'eau.

Les plaques sont révélées au nitrate d'argent ammoniacal contenant un peu d'acétate de sodium et chauffées à 110°. (Le réactif se prépare en ajoutant à environ

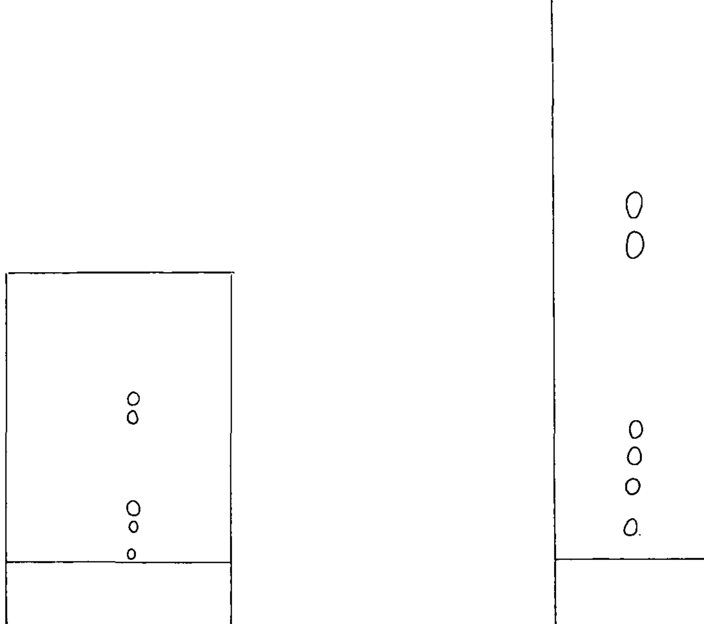

Fig. 1. Séparation de lactose, galactose, ribose, rhamnose et desoxyribose. Silène pur activé 15 h à 110°, solvant 2.

Fig. 2. Séparation de lactose, galactose, fructose, ribose, rhamnose et desoxyribose. Silène/Célite, double développement (8 puis, 16.9 cm).

* Silène EF, Columbia Chemical Division, Pittsburgh Plate Glass Co., Barberton, Ohio (U.S.A.).

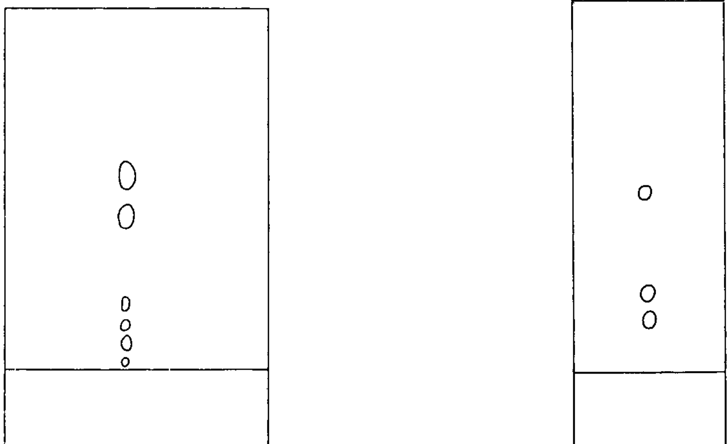

Fig. 3. Separation de maltose, glucose, xylose, ribose, rhamnose et desoxyribose. Silène/Célite, solvant 1.

Fig. 4. Séparation de galactose, ribose, rhamnose. Silène pur activé 1 h, solvant 2.

10 cm³ d'ammoniaque concentrée, 30 gouttes d'une solution aqueuse saturée de nitrate d'argent, et en complètant à 20 cm³ avec de l'éthanol distillé). Les taches sont très nettes, même dans le cas de séparation lente sur silène sans Célite; la sensibilité est fortement augmentée, réversiblement, en humectant les plaques. Voir Figs. 1–5.

Les réactifs préconisés par PASTUSKA[2] et par STAHL ET KALTENBACH[3] dans le cas des plaques de Kieselgel G ou de Kieselgur G sont insuffisamment sensibles sur plaques de silène. En développant les plaques à $+4°$ au lieu de $+25°$, on constate une diminution de 25 % de la hauteur à laquelle le solvant s'est élevé en 2 h; les R_F ne subissent pas de changement considérable.

Les plaques de silène semblent complèter utilement les techniques désormais classiques de STAHL[3] et de PASTUSKA[2] pour vérifier la pureté ou l'identité d'un sucre

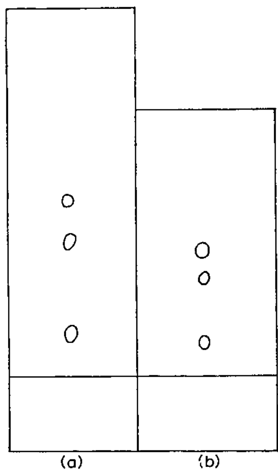

Fig. 5. Séparation de galactose, xylose et rhamnose. Silène/Célite, solvant 2. (a) à 25°, (b) à + 4°; temps de développement 2 h, 10 min.

inconnu, et pourraient servir d'étude préliminaire à une séparation préparative sur colonnes de silène. De plus, elles se recommandent par leur caractère particulièrement économique. Nous n'avons pas encore utilisé les plaques de silène pour la séparation d'autres catégories de produits.

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1 A. THOMPSON, dans R. L. WHISTLER ET M. L. WOLFROM (Editeurs), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York et Londres, 1962, p. 37.
2 G. PASTUSKA, *Z. Anal. Chem.*, 179 (1961) 427.
3 E. STAHL ET U. KALTENBACH, *J. Chromatog.*, 5 (1961) 351.

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J. Chromatog., 12 (1963) 413–415

Trennung im Harn vorkommender Zucker mit Hilfe der Dünnschichtchromatographie

Die Zucker werden in die schwächer polaren Phenylosazone übergeführt, die sich dünnschichtchromatographisch als Boratkomplexe auftrennen lassen.

10 ml Harn werden mit 0.4 g Phenylhydrazinhydrochlorid und 0.6 g Natriumacetat 30 Min. lang im siedenden Wasser erhitzt. Nach dem Kühlen mit fliessendem Wasser werden die ausgefallenen Kristalle abfiltriert, mit Wasser gewaschen und in einem Dioxan–Methanol-Gemisch (1:1 v/v) in der Kälte gelöst.

Als Vergleichslösungen werden 1 %ige Zuckerlösungen mit 0.2 g Phenylhydrazinhydrochlorid und 0.3 g Natriumacetat wie beschrieben behandelt.

Dünnschichtchromatographie. Sorptionsschicht (Trennschicht) (ausreichend für 5 Platten 200 × 200 mm): 30.0 g Kieselgur G (Merck) werden mit 60.0 ml einer 0.05 *M* wässrigen Natriumtetraboratlösung homogen angerieben und mit der Desaga-Grundausrüstung auf die Platten gestrichen. Letztere werden dann 30 Min. bei 80° getrocknet.

TABELLE I

R_F-WERTE DER IM HARN VORKOMMENDEN ZUCKER

Elutionsmittel: Chloroform–Dioxan–Tetrahydrofuran–0.1 *M* Natriumtetraborat (40:20:20:1.5 v/v)

Zucker als Phenylosazone	R_F
Glucose	0.39
Fructose	0.39
Arabinose	0.91
Galaktose	0.52
Lactose	0.02
Sorbose	0.21
Xylose	0.72
Ribose	0.91
Maltose	0.12

Elutionsmittel. 40.0 ml Chloroform p.a. und 40.0 ml Dioxan p.a. werden mit 1.5 ml 0.1 *M* wässriger Natriumtetraboratlösung unter kräftigem Umschütteln gemischt. Es wird mit Kammerübersättigung gearbeitet, Raumtemperatur ca. 23°, Laufzeit 20–25 Min., Trennstrecke 100 mm. Aufgetragene Zuckermenge 5–10 γ in 2 μl Lösungsmittel.

Da die gelbe Farbe der Zuckerosazone sehr schnell verblasst, müssen die Flecken nach dem Trocknen der entwickelten Platten bei Zimmertemperatur sofort markiert werden. Für die Resultate siehe Tabelle I und Fig. 1.

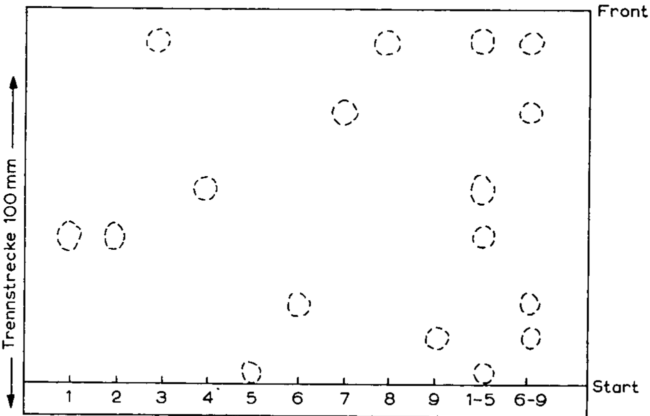

Fig. 1. Schematische Darstellung der getrennten Zuckerphenylosazone. 1 = Glucose; 2 = Fructose; 3 = Arabinose; 4 = Galaktose; 5 = Laktose; 6 = Sorbose; 7 = Xylose; 8 = Ribose; 9 = Maltose.

Glucose und Fructose bzw. Arabinose und Ribose unterscheiden sich nur in den C Atomen 1 und 2; sie geben daher gleiche Phenylosazone.

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J. Chromatog., 12 (1963) 415–416

A simple control unit for Lovelock ionisation detectors

The use of Lovelock ionisation detectors in gas chromatography is widespread because of their constructional simplicity and high sensitivity. With the simple type[1] of Lovelock detector the output consists of a standing current of about 10^{-8} A plus a current which is proportional to the concentration of the eluted compounds in the carrier gas as it passes through the detector and which ranges from 10^{-11} to 10^{-7} A. In the most common type of control unit for this detector these currents are passed through a resistor of about 25 kM Ω and the voltage produced fed into an infinite input and zero output resistance valve voltmeter of the type described by SCROGGIE[2]. This circuit exhibits high linearity and enables the full sensitivity of the detector to be utilised limited only by the noise inherent in these detectors, whilst at the same time

possessing characteristics which are virtually independent of value ageing. In addition, the control units for such detectors also provide a stabilised supply variable to various extents over the range 0–1500V for connection to the radioactive electrode.

Many applications of gas chromatography do not require such versatile and sophisticated instrumentation. There is little need for an absence of very slow "ageing" effects in the characteristics of the valve voltmeter since gas chromatographic columns age more rapidly than electronic valves. With a single operating voltage between 600 and 1000 V the detector usually has adequate sensitivity to most compounds, and except in special cases the highest sensitivity of the detector is not required.

A simplified amplifier and control unit suitable for feeding a 1 mV potentiometric recorder has been constructed in these laboratories. Fig. 1 shows the circuit

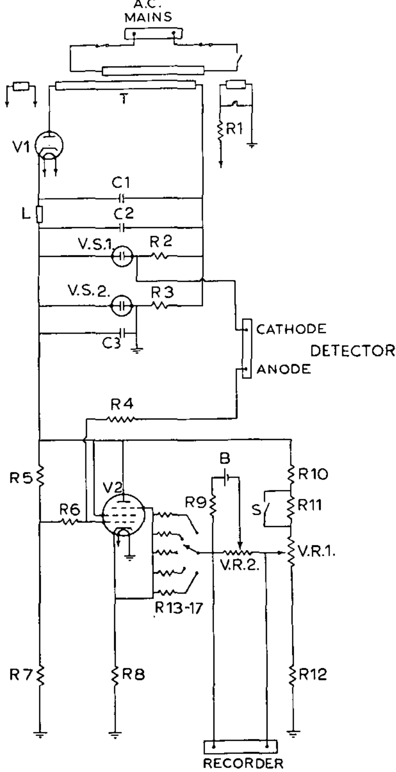

Fig. 1. Circuit diagram of control unit.

employed, the component values used being listed in Table I. In operation the circuit in the upper half of this diagram supplies 600 V, 800 V or 1000 V to the ionisation detector according to whether the V.S. 1. used is type SC1/600, SC1/800 or SC1/1000. For the first two of these corona stabilisers R2 is 1 M Ω, but is reduced to 470 k Ω when a SC1/1000 is used. In addition R3 and V.S. 2. provide the supply voltage for the single stage cathode follower amplifier shown in the lower part of the diagram.

The output current from the detector is fed through the linearising resistance R4 and the grid resistor R6 and drives the grid of V2 negative. In order to operate the

TABLE I

COMPONENTS VALUES FOR THE CIRCUIT SHOWN IN FIG. 1

T	Transformer with secondaries giving 6.3 V at 0.3 A, 6.3 V at 0.3 A, 500–0–500 V at 5 mA	V.S.2	85A2
L	20 Henry 20 mA choke	C1,C2	1 μF 1500 V.W.
V1	EY61	C3	32 μF 250 V.W.
V2	ME1400	V.R.1	50 k Ω 5 W W.W. potentiometer
V.S.1	G.E.C. Corona stabiliser type SC1/600, SC1/800 or SC1/1000, see text.	V.R.2	250 Ω 5 W W.W. potentiometer
		B	Mallory cell type RM-3
		S	Push to test switch
R1	5 Ω 1 W	R10	280 k Ω 1/2 W
R2	1 M Ω 1/4 W (see text)	R11	10 k Ω 1/4 W
R3	350 k Ω 2 W	R12	120 k Ω 1/2 W
R4	10^9 Ω Welwyn ± 20%	R13	3 M Ω 1/4 W
R5	470 k Ω 1/4 W	R14	1 M Ω 1/4 W
R6	10^8 Ω Welwyn ± 20%	R15	330 k Ω 1/4 W
R7	220 k Ω 1/4 W	R16	100 k Ω 1/4 W
R8	100 k Ω 1/4 W	R17	33 k Ω 1/4 W.
R9	470 k Ω 1/4 W		

triode connected semi-electrometer pentode type ME1400 on the linear part of its characteristics, R6 is connected to the positive potential appearing at the junction of R5 and R6. Input voltages to the grid of V2 may change by as much as 5 V without peak doubling being shown by the detector, and a voltage of the same order as the input voltage appears at the cathode of V2, in addition to its D.C. potential which is balanced against the voltage provided by V.R.1

An appropriate fraction of the signal voltage appearing at the cathode of V2 is selected and fed to the recorder by R's 13–17 and V.R. 2. which enable the sensitivity to set at 1, 3, 10, 30 and 100 times its lowest value. With potentiometric recorders requiring more than 1 mV for full scale deflection the value of V.R.2. must be increased and with current recorders this resistance must be omitted from the circuit.

When setting up, the voltage produced by the standing current is balanced out and the recorder set to zero by adjustment of V.R.1. Battery B and R9 provide a counter current through V.R.2. to produce a potential which fixes the position of the baseline at any desired level according to the setting of V.R.2., this baseline position being independent of the sensitivity employed. The push switch S and R11 are included in the circuit to enable a mark to be produced on the record when required.

In use, the output of this unit on the most sensitive range drifts 0.1–0.05 mV in the first quarter hour after switching on, and thereafter the drift is less than ± 0.01 mV per hour, the noise level on this range being equivalent to ± 0.01 mV. These values are of course reduced when the less sensitive ranges are employed.

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[1] J. E. LOVELOCK, in R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, London, 1960, p. 16.
[2] M. G. SCROGGIE, *Wireless World*, 58 (1952) 14.

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A simple device for the simultaneous elution of multiple papergrams

When ion exchange or column chromatographic methods are not satisfactory for the isolation and separation of biochemical compounds, paper chromatography or paper electrophoresis often provide a suitable method of separation. The workable amounts, however, are limited in these cases so that a large number of papergrams must be obtained before sufficient amounts can be isolated for investigative purposes.

Fig. 1 illustrates a simple and convenient device that permits the simultaneous elution of several paper strips, and the collection of eluates into a single container. The device consists of a glass rod bent into a hexadecagon of approximately 5 in. across and $^3/_4$ in. sides. The rod can support up to 16 paper wicks $^1/_2$ to $^5/_8$ in. in width.

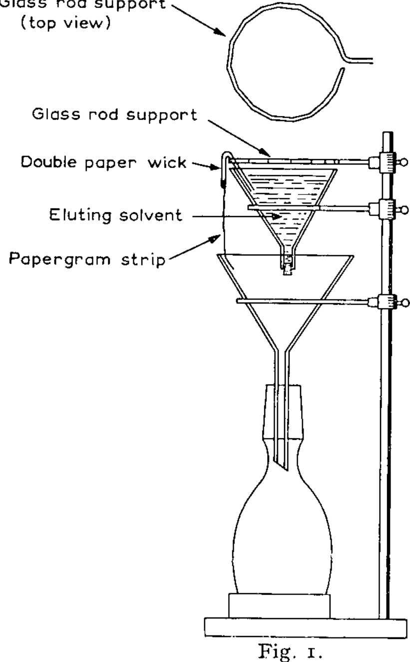

Fig. 1.

The eluting solvent is contained in a 4 in. funnel stoppered at the stem, and held directly below the glass rod with an iron ring. The papergrams strips are placed between the two paper wicks previously wetted with the eluting solvent, and are allowed to hang inside the 6 in. funnel so that the eluate can be collected in a single container. Adjustment for sets of strips of different length can be made by lowering or raising the larger funnel. The rate of elution can be controlled by increasing or decreasing the length of the double wick projecting out of the glass rod. The entire assembly can be inserted in a 12 gallon rectangular jar and maintained covered to prevent excessive evaporation.

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Color reactions of 4-alkylresorcinols and some naturally occurring phenolics with Ehrlich reagent

Ehrlich reagent, consisting of an acidic solution of *p*-dimethylaminobenzaldehyde (PDAB), is generally used for the detection of indole and pyrrole derivatives[1–3]. However, MORTON[2] in 1946 reported that certain phenols produce colors comparable to indoles under conditions of Ehrlich test. Thereafter studies on a number of phenolic compounds indicate that several resorcinols and phloroglucinols give the color tests with the reagent[4–7]. Recently ACHESON AND TURNER[8] have reported that a resorcinol requires a free 4 or 6 position, and it must not possess an uncompensated carbonyl (deactivating) group, if it is to give color with the reagent.

In our work we have confirmed the finding that the presence of a carbonyl group in resorcinols inhibits the reaction. However, study of several 4-alkyl-substituted compounds has shown some interesting results. Although 4-ethyl- and 4-*n*-propylresorcinols show color formation, spot tests on paper on 4-*n*-hexyl-, 4-*n*-dodecyl-, 4-*n*-hexadecyl-, and 4-*n*-octadecylresorcinols indicate that with an increase in alkyl chain there is an inhibition of color reaction. Comparative color tests on 4-*n*-hexyl-, 4-cyclohexyl-, and 4-benzylresorcinols have shown that the third compound gives a more intense coloration than either of the other two. On the other hand 4-*n*-hexylresorcinol yields even a weaker reaction than the corresponding cyclohexyl derivative.

Examination of several naturally occurring phenolic compounds that contain a resorcinol or phloroglucinol moiety in their molecule has been made. Spot tests on

TABLE I

COLOR REACTION OF PHENOLIC COMPOUNDS WITH EHRLICH REAGENT

No.	*Compound*	*Color observed*	*Amount of test compound* (μg)*	*Distinguishing compound(s) No.*
1	Sesamol	Blue**	1	—
2	Cannabidiol	Bluish green**	1	3, 4
3	Cannabinol	Pale pink-brown	5	2
4	Tetrahydrocannabinol***	Pale bluish brown	5	2
5	D-Catechin	Violet**	1	6–9
6	Hesperetin	Orange-yellow	5	5
7	Quercetin	Yellow	5	5
8	Quercitrin	Yellow	5	5
9	Rutin	Yellow	10	5
10	Usnic acid	Pale pink	100	—
11	Aspidin	Pale yellow	100	15
12	Albaspidin	Pale yellow	100	15
13	Desaspidin	Pale yellow	100	15
14	Flavaspidic acid	Pale yellow	100	15
15	Butyrylfilicinic acid	Orange-red**	1	11–14
16	Phloridzin	Light pink-yellow	5	17
17	Phloretin	Pink**	1	16

* Spot tests on five different quantities 1, 5, 10, 50, and 100 μg of each compound were performed and lowest amount of the compound that gave color reaction is recorded.

** These compounds show color even at room temperature after applying the reagent on the spot.

*** Synthetic tetrahydrocannabinol (m.p. 62–63°C) provided by Drs. SIEPER AND KORTE was used. In the compound the alicyclic double bond is conjugated with the olivetol ring.

paper were performed with five different quantities of each of these compounds. 2 λ of 0.5 % PDAB in ethyl alcohol containing 1 % hydrochloric acid was applied on the 3 mm diameter of each compound and the paper kept in an oven at 100° for 1 min. The compounds examined and the results of these tests are recorded in Table I.

The results recorded in Table I indicate the usefulness of Ehrlich reagent for detecting small amounts, at least 1 μg, of compounds numbers 1, 2, 5, 15, and 17. The usefulness of the reagent for distinguishing these plant phenolics from those listed that are closely related is also significantly revealed.

More details of these preliminary studies on the compounds reported here and other phenols tested will be published.

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1 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd. Ed., Academic Press, New York, 1958, p. 318.
2 A. A. MORTON, *The Chemistry of Heterocyclic Compounds*, McGraw-Hill, New York, 1946, p. 68.
3 F. FEIGL, *Spot Tests in Organic Analysis*, 6th Ed., Elsevier, Amsterdam, 1960, p. 289.
4 R. M. ACHESON, R. M. PAUL AND R. V. TOMLISON, *Can. J. Biochem. Physiol.*, 36 (1958) 295.
5 C. STEELINK, *Nature*, 184 (1959) 720.
6 E. G. MCGEER, M. C. ROBERTSON AND P. L. MCGEER, *Can. J. Biochem. Physiol.*, 39 (1961) 605.
7 K. N. F. SHAW AND J. TREVARTHEN, *Nature*, 182 (1958) 797.
8 R. M. ACHESON AND I. TURNER, *J. Chromatog.*, 7 (1962) 520.

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A technique for the recovery of compounds from thin-layer chromatograph strips for infrared analysis

An examination of technical grade rotenone by thin layer chromatography (TLC) revealed at least three spots. Since R_F values alone cannot be considered conclusive proof for the identity of a compound, a supplementary procedure was necessary to determine which of the three spots was rotenone. A simple technique was devised to collect the components from the developed TLC strip and to confirm their identity by infrared spectrophotometry. This procedure, which should be applicable to other compounds resolved by TLC, is reported below.

The amount of compound necessary for analysis may be within the range of 10–50 μg. To aid in locating non-fluorescent spots, small amounts (0.5 % each) of

zinc silicate and zinc cadmium sulfide were incorporated in the silica gel layer used as the support on the strips. After development of the strip in a suitable solvent mixture, the rotenone spots were located with the aid of an ultraviolet lamp and the area was marked above and below the spot by means of a knife blade. The silica gel layer

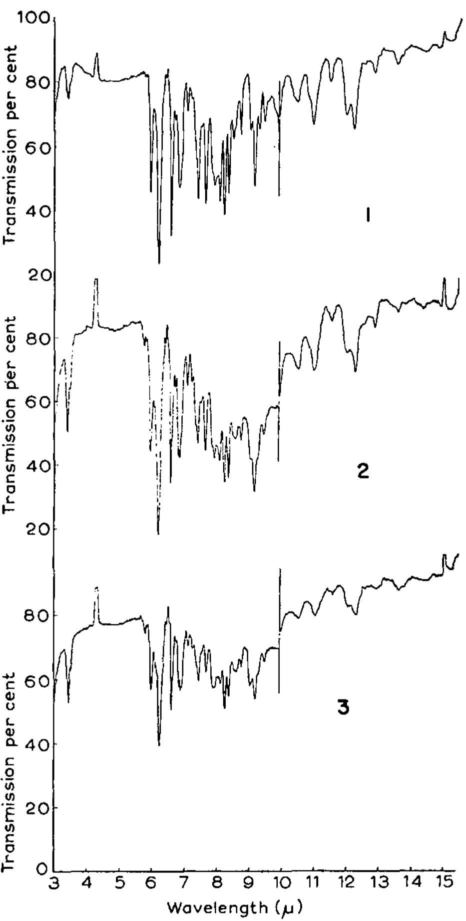

Fig. 1. Infrared spectra of rotenone. 1. Recrystallized rotenone m.p. 161–163°. 2. Recrystallized rotenone obtained from TLC strip. 3. Rotenone isolated from technical rotenone (Derris root) by TLC.

within these marked boundaries was scraped into a 15 ml centrifuge tube containing 3 ml spectro grade ethyl acetate. The contents of the tube were mixed thoroughly and centrifuged. The supernatant liquid was transferred by means of a pipette to a 10 × 30 mm stainless steel vial and concentrated with a gentle stream of air. It is important that the supernatant liquid be removed carefully from the centrifuge tube to avoid any possible agitation that might produce turbidity and contaminate the liquid with some solids from TLC residue. Silica gel contamination will void the true infrared spectra of the compound. The vial was then placed in an Abderhalden apparatus for one hour to remove the last traces of solvent from the residue. A

known amount of spectro grade potassium bromide was added to the vial and mixed mechanically with the residue. The mixture was transferred to a $1^1/_2$ mm die, a pellet was pressed, and the spectrum of the pellet was recorded with an infrared spectrophotometer equipped with a 6 × beam condenser. A comparison of the spectrum with a known spectrum of rotenone (Fig. 1) showed that the compound isolated from the TLC strip was rotenone and illustrated that the technique was feasible for the isolation of a pure compound.

Work is being continued on separation and isolation of other rotenoid compounds.

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Paper chromatography of alkaloids of tall fescue hay

The alkaloids of tall fescue hay (*Festuca arundinacea* Schreb.) have been examined as a part of our work on the etiology of a cattle disease known as fescue foot[1]. Perloline has been isolated from tall fescue[2], and the presence of ergot-like alkaloids has been indicated by color tests[3]. However, no references have been found on the systematic separation of fescue alkaloids. This communication describes the separation of nine alkaloids by paper chromatography, and a method for isolating certain individual alkaloids.

Experimental

One to several hundred grams of toxic tall fescue hay were extracted by refluxing with 80 % aqueous ethanol (v/v). This extract was concentrated under reduced pressure to remove the alcohol, made basic with aqueous NaOH to pH 11, and exhaustively extracted with chloroform. The alkaloids were then extracted from the chloroform into *N*/10 HCl. The alkaloids were cycled again between chloroform and *N*/10 HCl. The final acidic solution was concentrated to a syrup at 40° under vacuum. Yield of crude alkaloids was about 0.1 % of the dry hay.

Whatman No. 1 paper * was washed with *N*/100 HCl and air dried at room temperature. Guidelines were drawn before washing so that development would be in the machine direction. Several hundred micrograms of crude alkaloid were placed on a spot, but if only single components were present, much smaller amounts were used (10–100 μg). Chromatograms were usually conditioned for 6–16 h with freshly prepared solvent (*n*-butanol–glacial acetic acid–water (10:1:3)) and developed by the descending procedure. The solvent front travels about 40 cm in 16 h at 25–27°. The developed chromatograms were air dried at 25–27°.

* Mention of trade names does not imply endorsement by the Department of Agriculture over similar materials not so named.

The dried chromatograms were scanned, first under white light and then under a long wave (3660 Å) ultraviolet lamp. The solvent front and any spots that fluoresced or absorbed were marked. The chromatograms were then sprayed with a color reagent to detect the remaining spots. Two types of color reagents were used, either a reagent which detects most alkaloids or reagents designed to form colors with specific functional groups.

Potassium iodoplatinate[4] was the best general alkaloid reagent examined and was ordinarily used. This reagent forms spots of different colors with the various alkaloids. The background color may be removed by washing the chromatograms with water after the reagent has dried. The spots of most alkaloids are not soluble in water. The limit of detection for fescue alkaloids is in the order of a few parts per million. Special reagents such as ninhydrin, ammoniacal silver nitrate, and diazotized *p*-nitroaniline[5] were used to detect reduced nitrogen groups, reducing compounds, and phenolic groups, respectively. Color development, except in the case of ninhydrin, was in air at room temperature.

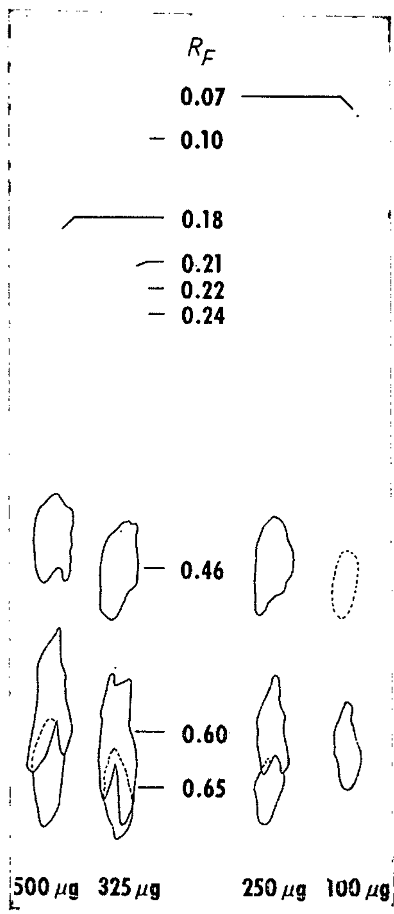

Fig. 1. Paper chromatogram of tall fescue alkaloids sprayed with potassium iodoplatinate. Conditions for development are given in the text.

For preparative paper chromatography, Whatman No. 3 MM paper was used without washing and was developed as described. Solutions were streaked across the chromatograms until the concentration was about 1 mg/cm. After the chromatograms were developed, areas containing particular alkaloids were cut out and eluted with $N/10$ HCl according to the method of DENT[6].

Results

There are at least nine different alkaloids in fescue (Fig. 1 and Table I). The major component is the alkaloid of R_F 0.10, representing approximately one-half of the

TABLE I

FESCUE ALKALOIDS DETECTED IN CRUDE EXTRACTS BY PAPER CHROMATOGRAPHY

R_F	*Color with potassium iodoplatinate*
0.07	Light gray
0.10	Blue-gray
0.18	Light gray
0.21	Light gray
0.22	Pink
0.24	Blue-gray
0.46	Yellow to tan*
0.60	No color**
0.65	Violet

* Yellow in white light.
** Spot fluoresces light blue in ultraviolet light.

alkaloidal material separated from tall fescue. Perloline appears at R_F 0.46. It was identified by its fluorescence, color, and ultraviolet spectrum[7,8].

The alkaloids of toxic tall fescue hay were compared with alkaloids in toxic fresh forage, non-toxic fescue hay, and rye grass. Alkaloids of the three fescue samples

TABLE II

R_F VALUES AND COLOR REACTIONS OF COMMERCIAL ALKALOIDS

Alkaloid	R_F	*Color with potassium iodoplatinate*
Caffeine	—	None
Theobromine	—	None
Theophylline	—	None
Ephedrine	—	None
Cacotheline	0.09	Tan
Brucine	0.43	Blue
Berberine	0.46	Tan
Pilocarpine	0.33	Gray
Quinine	0.52	Purple
Harmol	0.60	Light blue
Harmine	0.57	Violet
4-Hydroxy-L-proline	—	None
Ergotamine	0.77	None*

* Reacts if HCl fumes are present.

were chromatographically identical. The major alkaloids of rye grass were chromatographically identical to those in fescue. However, the alkaloid of R_F 0.65 was not detected, and there were small amounts of additional alkaloids in rye grass with an R_F value less than 0.07. When the alkaloid extract of a sample of fescue hay diseased with *Stemphyllium* sp. fungus was chromatographed, the alkaloid of R_F 0.10 and one or more of the alkaloids of R_F 0.18–0.24 were not present.

No similarities were found when fescue alkaloids were compared with a number of commercial alkaloids (Table II).

Chemical characterization of the major alkaloid, to be published elsewhere, might shed some light on the toxicity problem of tall fescue and provide the key to the structure of fescue alkaloids.

Acknowledgement

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1 S. G. YATES, *Econ. Botany*, 16 (1962) 295.
2 R. H. F. MANSKE, *The Alkaloids, Chemistry and Physiology*, Vol. V, Academic Press, Inc., New York, 1955, p. 316.
3 D. D. MAAG AND J. W. TOBISKA, *Am. J. Vet. Res.*, 17 (1956) 202.
4 G. NADEAU, *Clin. Chem.*, 2 (1956) 347.
5 H. G. BRAY, W. V. THORPE AND K. WHITE, *Biochem. J.*, 46 (1950) 271.
6 C. E. DENT, *Biochem. J.*, 41 (1947) 240.
7 I. REIFER AND N. O. BATHURST, *New Zealand J. Sci. Technol.*, 24B (1943) 151.
8 F. B. SHORLAND, *New Zealand J. Sci. Technol.*, 24B (1943) 159.

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Book Reviews

Thin Film Chromatography, by E. V. TRUTER, Cleaver-Hume Press Ltd., London, 1963, xii + 205 pages, price 37/6.

The book under review consists of about 100 pages describing thin-layer techniques and quantitative methods and about 80 pages of applications of thin-layer chromatography to various classes of compounds.

The general part will be found adequate both for beginners and as a reference work. The applications are, however, only a selection and it is felt that the English edition of the book by E. STAHL is by far more detailed and comprehensive.

The author suggests *Thin Film Chromatography* as a more apt name for what is known as thin-layer chromatography. It seems too late to change a name which is already so well established. Furthermore STAHL (at the symposium on thin-layer chromatography, May 1963, Rome) explained his choice of the term "thin layer", that when talking about thin films one thinks rather also of the flexible nature of films while thin layer suggests correctly the rigid nature of the adsorbent layer employed.

The book contains 282 references, an author and a subject index and only minor printing errors were noted by the reviewer.

J. Chromatog., 12 (1963) 427

Microchemical Techniques, Proceedings, 1961—International Symposium on, edited by N. D. CHERONIS, Interscience Publishers, New York, 1962, xviii + 1181 pages, price 300/—.

There are few valid excuses for publishing the proceedings of a symposium almost two years late. Unfortunately one of them, namely the death of the editor, applies to this volume.

The symposium was attended by numerous people thus the proceedings which give plenary lectures, original papers and discussions in full cover 1181 pages.

About 100 pages are papers on chromatographic techniques of which some have not lost their value yet. The round table discussion on chromatography is unfortunately reported in only one page and seems of little interest.

As the book contains about as much original material as an average yearly volume of a journal of analytical chemistry it should be available in all libraries. It is a pity that the publishers have imposed a higher price than for a subscription to a journal, thus the price being out of proportion to the value and much higher than the average for scientific books.

J. Chromatog., 12 (1963) 427

Bibliography Section

Paper Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

GREEN, J. AND MARCINKIEWICZ, S.: Paper chromatography and chemical structure. V. Tautomerism. The determination of tautomeric equilibrium by paper chromatography. Thienol and *p*-nitrosophenols. *J. Chromatog.*, 10 (1963) 354–365 — R_F values of 22 compounds.

GREEN, J. AND MARCINKIEWICZ, S.: Paper chromatography and chemical structure. VIII. Hyperconjugation. *J. Chromatog.*, 10 (1963) 389–391.

MARCINKIEWICZ, S. AND GREEN, J.: Paper chromatography and chemical structure. VI. Tautomerism and intramolecular hydrogen bonding in the same molecule. *o*-Nitrosophenols. *J. Chromatog.*, 10 (1963) 366–371 — 28 compounds tabulated.

MARCINKIEWICZ, S. AND GREEN, J.: Paper chromatography and chemical structure. VII. The separation of *meta*- and *para*-derivatives of benzene. *J. Chromatog.*, 10 (1963) 372–388 — extensive R_F tables.

3. TECHNIQUES I

ANDERSON, J. R. A., LARK, P. D. AND MARTIN, E. C.: Solvent effects in inorganic chromatography. I. Factors affecting the adsorption front. *J. Chromatog.*, 10 (1963) 338–346.

FORSS, D. A. AND RAMSHAW, E. H.: The chromatography of 2,4-dinitrophenylhydrazones on acetylated paper. *J. Chromatog.*, 10 (1963) 268–271 — alkanones, alkanals, alkenals, alkandienals.

KNY, L.: Die direkte fluorometrische Bestimmung der Digitalisglykoside am Papierchromatogramm. I. Teil. *Pharmazie*, 18 (1963) 209–222 — R_F values of 20 steroid glycosides.

LUGG, J. W. H.: Aspects of the direct photometry of substances distributed on translucent sheets. *J. Chromatog.*, 10 (1963) 272–280.

MARTIN, E. C.: The distribution of acids, water and butan-1-ol on chromatograms made by the ascending technique. *J. Chromatog.*, 10 (1963) 347–353.

RITCHIE, A. S.: Some factors influencing the constancy of R_F values in paper chromatography under Australian field conditions. *J. Chromatog.*, 10 (1963) 281–283 — 24 metal ions.

STAMM, H. H. AND SCHROEDER, H. J.: Chromatographische Trennungen von Radionukliden mit Eisen(III)-hydroxid auf Papierträger. *J. Chromatog.*, 10 (1963) 392–395 — P, S, Cl, Y, Sr, Cs.

6. ALCOHOLS

FREUDENBERG, K. AND GEIGER, H.: Pinoresinolid und andere Zwischenprodukte der Ligninbildung. *Chem. Ber.*, 96 (1963) 1265–1270 — R_F values of 11 substances.

HENNIES, J. AND ECKERT, B.: Die Anwendung der Papierchromatographie bei der Untersuchung von Kosmetica. I. Der Papierchromatographische Nachweis von mehrwertigen Alkoholen. *Pharmazie*, 18 (1963) 127–128.

PATTERSON, S. J.: The detection and determination of glycerol in tobacco. The use of paper and cellulose-column chromatography for determining glycerol in the presence of sugars. *Analyst*, 88 (1963) 387–393.

STANISHEVSKAYA, E. M.: (Determination of the specific radioactivity of phytol by paper chromatography). *Vestsi Akad. Nauk Belorusk. SSR, Ser. Biol. Nauk*, No. 2 (1962) 62–70; *C.A.*, 58 (1963) 740h.

7. PHENOLS

KAWASHIRO, I., KAWATA, K. AND HOSOGAI, Y.: Studies on the detection of food adjuncts. II. Separation and identification of antioxidants by reversed-phase paper chromatography. *Eisei Shikensho Hokoku*, 77 (1959) 175–177; *C.A.*, 57 (1962) 15561a.

WAGNER, G. AND BÖHM, M.: Über die Darstellung von Resacetophenongalaktosiden, Resacetophenonlactosiden und gemischten Resacetophenon-bisglykosiden. *Pharmazie*, 18 (1963) 202–206 — R_F values of 9 glycosides.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

BIOL, H. AND FOULONNEAU, C.: (The diglucoside 3,5-paeonidol in the genus *Vitis*). *Ann. Technol. Agr.*, 10 (1961) 345–350; *C.A.*, 57 (1962) 13015c.

BOPP, M. AND MATTHISS, B.: Über die Zusammenhang der Biogenese von Anthocyan und Leukoanthocyan. *Z. Naturforsch.*, 17b (1962) 811–818.

REYNOLDS, T., ATTERTON, R. M., KIRSOP, B. H. AND POOL, A. A.: Polyphenols in malting and brewing. *European Brewery Conv., Proc. 8th Congr., Vienna,* 1961, p. 267–277; *C.A.*, 57 (1962) 15616d.

TRONCHET, J.: (Comparison by paper chromatography of the flavonoids of control plants and of those treated with gibberellin (*Mercuriais annua* and *Lactuca saligna*).). *Ann. Sci. Univ. Besancon, Botan.*, (2) No. 17 (1961) 83–88; *C.A.*, 57 (1962) 14218c.

9. OXO COMPOUNDS

DUQUENOIS, P. AND HAAG-BERRURIER, M.: (Composition of *Hieracium pilosella*). *Boll. Chim. Farm.*, 101 (1962) 347–353; *C.A.*, 57 (1962) 17072a.

KEGEL, L. P., HENNINGER, M. D. AND CRANE, F. L.: The new quinones from chloroplasts. *Biochem. Biophys. Res. Commun.*, 8 (1962) 294–298; *C.A.*, 57 (1962) 12900d.

YAMAGUCHI, M.: Pigments of marine animals. XIV. Paper chromatography of polyhydroxynaphthoquinones. *Mem. Fac. Sci., Kyushu Univ.*, C4 (1961) 189–192; *C.A.*, 57 (1962) 15623g.

10. CARBOHYDRATES

BUCHANAN, J. G. AND SCHWARZ, J. C. P.: Methyl 2,3-anhydro-α-D-mannoside and 3,4-anhydro-α-D-altroside and their derivatives. Part I. *J. Chem. Soc.*, (1962) 4770–4777 — R_F values of 9 anhydro sugars.

CARLES, J.: (New sugars from the vine and from wine). *Compt. Rend.*, 255 (1962) 761–762; *C.A.*, 57 (1962) 14292e.

CURYLO, J.: (Chromatographic separation of carbohydrates from nectar and leaf honey). *Pszczel. Zeszyty Nauk*, 6, No. 1 (1962) 1–14; *C.A.*, 57 (1962) 14251e.

CURYLO, J.: (Determination of reducing sugars in pine-leaf honey with *p*-aminoazobenzene). *Pszczel. Zeszyty Nauk*, 6, No. 1 (1962) 15–24; *C.A.*, 57 (1962) 14251g.

JONES, J. K. N. AND TURNER, J. C.: 5-Acetamido-5-deoxy-L-arabinose: a sugar derivative containing nitrogen as the heteroatom in the ring. *J. Chem. Soc.*, (1962) 4699–4703 — R_F values of 7 sugar derivatives.

KRYM, I. YA.: (Use of partition chromatography for the determination of carbohydrates in soils). *Pochvovedenie*, No. 6 (1962) 101–103; *C.A.*, 57 (1962) 12930a.

LEVKOVICH, A. A.: (Detection of carbohydrates by ascending paper chromatography). *Izv. Irkutsk. Nauchn.-Issled. Protivochumn. Inst. Sibiri i Dal'n. Vost.*, 23 (1960) 113–116; *C.A.*, 59 (1963) 740a.

PEYNAUD, E., GUIMBERTEAU, G. AND DOMERCQ, S.: (Application of sugar chromatography for identifying lactic acid bacteria in wine). *Mitt. (Klosterneuburg), Ser. A, Rebe Wein*, 12 (1962) 183–192; *C.A.*, 57 (1962) 15615h.

RENDIG, V. V. AND MCCOMB, E. A.: Pentoses as precursors of heptuloses in plants. *Arch. Biochem. Biophys.*, 99 (1962) 409–413.

SUDA, M.: Mucopolysaccharides of the stroma of the cornea and on incorporation of inorganic sulfate-S^{35} into polysaccharide fractions in rabbit cornea. *Biochem. Med. Mucopolysaccharides*, (1962) 213–219; *C.A.*, 57 (1962) 14320d.

WYSS-HUBER, M., STRIEBEL, H., WEISS, E. AND GEIGY, R.: Paper chromatographic determination of various sugars and spectrophotometric estimation of glycogen in extracts of *Glossina*, reduviids, and *Ornithodorus moubata*. *Acta Trop.*, 18 (1961) 46–57; *C.A.*, 57 (1962) 13021i.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

ARATA, L. AND MANGIOLA, S.: (The metabolism of salicylic acid in man. I. Determination in the urine). *Progr. Med.*, 18 (1962) 229–234; *C.A.*, 57 (1962) 16987b.

BIOL, H. AND FOULONNEAU, CH.: (Extraction and assay of sorbic acid in wine). *Ann. Technol. Agr.*, 11 (1962) 131–144; *C.A.*, 57 (1962) 14292b.

HENNIG, K. AND BURKHARDT, R.: (Chromatographic separation of oakwood extracts and their proof in wines). *Weinberg Keller*, 9 (1962) 223–231; *C.A.*, 57 (1962) 14294b.

JOHNSEN, O. AND HADLAND, G.: (Rapid determination of butyric acid and other volatile fatty acids in milk, cheese and silage by circular paper chromatography). *Meieriposten*, 49 (1960) 229–241; *C.A.*, 57 (1962) 12966b.

KOIDE, F. AND SHIMURA, K.: 3-Phosphoglyceric acid dehydrogenase from the silk gland. *J. Biochem. (Tokyo)*, 52 (1963) 302–303.

KRAJCIOVA, V., CIZMAR, J. AND KRCMERY, V.: (Chromatographic determination of fatty acids in endotoxins and in whole cells of *Salmonella choleraesuis*). *Biologia*, 17 (1962) 148–152; *C.A.*, 57 (1962) 17169h.

KUTÁČEK, M., ROSMUS, J. AND DEYL, Z.: New methods of chromatographic separation of gibberellins A_1 and A_3. *Biol. Plant. Acad. Sci. Bohemoslov.*, 4, No. 3 (1962) 226–231; *C.A.*, 57 (1962) 16985d.

MARTELLI, H. L.: (Separation of structural analogs of Krebs cycle dicarboxylic acids by paper chromatography). *Anais Assoc. Brasil. Quim.*, 20 (1961) 21–28; *C.A.*, 58 (1963) 738d.

OBATA, Y. AND MATANO, K.: Flavor of natto. I. Identification of volatile acid compounds by paper chromatography. *Nippon Nogei Kagaku Kaishi*, 33 (1959) 567–569; *C.A.*, 57 (1962) 15569f.

POHLOUDEK-FABINI, R. AND PAPKE, E.: Über die Synthese einiger 2-Alkyl-3-hydroxychinoxaline aus α-Ketosäuren und *o*-Phenylendiamin und ihr papierchromatographisches Verhalten. *Pharmazie*, 18 (1963) 273–277.

TAKANO, T., ITAYA, T. KAMIHARA, T. AND NAKANO, S.: Lipid metabolism in experimental pulmonary edema. *Kanazawa Daigaku Kekkaku Kenkyusho Nempo*, 19 (1961) 217–223; *C.A.*, 57 (1962) 15697d.

WUCHERPFENNIG, K. AND BRETTHAUER, G.: (Fruit acids in apple and pear juice). *Deut. Lebensm.-Rundschau*, 58 (1962) 190–192; *C.A.*, 57 (1962) 17152e.

13. STEROIDS

AINSWORTH, L., CARTER, A. L. AND COMMON, R. H.: A chromatographic study of some conversion products of estrone-16-C^{14} in the urine and feces of the laying hen. *Can. J. Biochem. Physiol.*, 40 (1962) 123–135; *C.A.*, 57 (1962) 14363i.

KNIGHTS, B. A., ROGERS, A. W. AND THOMAS, G. H.: 5α-Pregnane-3α,6α,20α-triol, a metabolite of progesterone in the rabbit. *Biochem. Biophys. Res. Commun.*, 8 (1962) 253–258; *C.A.*, 57 (1962) 13120c.

14. STEROID GLYCOSIDES

KNY, L.: Die direkte fluorometrische Bestimmung der Digitalisglykoside am Papierchromatogramm. 2. Teil. *Pharmazie*, 18 (1963) 295–307.

16. NITRO AND NITROSO COMPOUNDS

AMATI, A. AND BARBERO, L.: (Detection of chloropicrin in wines by paper chromatography). *Ital. Vinicola Agrar.*, 52 (1962) 228–231; *C.A.*, 57 (1962) 17206b.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

RAGNO, I. AND PINTO, R.: (Tryptophan–nicotinic acid metabolism in liver cirrhosis in man). *Biochim. Appl.* 8 No. 6 (1961) 354–359; *C.A.*, 57 (1962) 15703h.

18. AMINO ACIDS

AVSHALUMOVA, A. D.: (Determination of the freshness of meat by distributive paper chromatography). *Materialy 7-oi (Sed'moi) Nauchn. Konf. po Infekts. i Invazion. Zabolevaniyam Sel'skokhoz. Zhivotnykh, Moscow, Sb.*, (1961) 80–83; *C.A.*, 57 (1962) 17149i.

BACLIONI, C., FASELLA, P., TURANO, C. AND SILIPRANDI, N.: (Tryptophan metabolism in normal rat). *Giorn. Ital. Biochim.*, 9 (1960) 10–22; *C.A.*, 57 (1962) 14263h.

BAILEY, J. L.: Paper chromatography of amino acids and peptides. In: *Techniques in Protein Chemistry*, Elsevier, Amsterdam, 1962, pp. 1–36 — a review with 55 references.

BERGAMASCHI, M., CHIESA, F., OBEROSLER, R. AND AGUGGINI, G.: (Behavior of free amino acids in milk from healthy cows). *Arch. Vet. Ital.*, 12 (1961) 9–12; *C.A.*, 57 (1962) 15653d.

COHEN, S., THOM, E. AND BENDICH, A.: Amides and peptides derived from 6-trichloromethylpurine and amino acids. *Biochemistry*, 2 (1963) 176–181 — R_F values of 40 compounds.

DAMBERGA, B.: (Chemical characteristics of some protein hydrolyzates). *Probl. Gematol. i Pereliv. Krovi*, 7, No. 8 (1962) 49–55; *C.A.*, 58 (1963) 690f.

FOODEN, J.: Urinary amino acids of nonhuman primates. *Zoologica*, 46 (1961) 167–180; *C.A.*, 57 (1962) 15672c.

FUČÍK, M. AND BOSÁKOVÁ, A.: (Proteins and amino acids in the gastric juice of man). *Acta Univ. Carolinae, Med. Suppl.*, No. 14 (1961) 125–135; *C.A.*, 57 (1962) 17244f.

KHARAT'YAN, A. M.: (Free amino acids in blood serum and urine of patients with anemias of gastrointestinal origin). *Patol. Kishechnika i Sistema Krovi, Sb. Nauchn. Rabot Kafedry Propedevtiki Vnutr. Boleznei Pediatr. i Sanit. Fak. Tashkentsk. Gos. Med. Inst.*, (1961) 154–176; *C.A.*, 57 (1962) 15694c.

MASSI, O.: (Chromatographic research on release of amino acids during defrosting of meat). *Atti Soc. Ital. Sci. Vet.*, 13 (1959) 380–382; *C.A.*, 57 (1962) 12967a.

MIRZOYAN, A. T. AND RADZHABOVA, T. K.: Qualitative determination of free amino acids in green tea leaves by paper chromatography. *Dokl. Akad. Nauk Azerb. SSR*, 18, No. 5 (1962) 41–43; *C.A.*, 57 (1962) 16986a.

PANT, R. AND AGRAWAL, H. C.: β-Naphthoquinone-4-sodium sulphonate as a specific spray reagent for the identification of some amino acids and their isomers. *Naturwissenschaften*, 50 (1963) 331.

SALVADORI, P., BIANCHI, B. AND CAVALLI, V.: (Chromatographic research of free amino acids in milk treated at different temperatures and its relation to growth of lactic bacteria). *Latte*, 36 (1962) 529–531; *C.A.*, 57 (1962) 12965g.

SHINDE, V. A. AND VERMA, S. C. L.: Paper chromatography of free amino acids from the serum of blood donors. *J. Anat. Soc. India.*, 10 (1961) 39–40; *C.A.*, 57 (1962) 15646c.

STEWART, I.: Determination of ornithine, lysine, arginine, citrulline and histidine. *J. Chromatog.*, 10 (1963) 404–407.

TOMITA, T.: (Presence of δ-hydroxylysine in extracts of Japanese centipedes (*Scolopendra subspinipes*). Paper chromatographic separation of free amino acids and isolation of δ-hydroxylysine). *Z. Physiol. Chem.*, 328 (1962) 266–268; *C.A.*, 57 (1962) 12840c.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

AVALOS, N. ADRIAZOLA AND BERNARDO E. FERNANDEZ: (Synthesis and chromatographic analysis of the dinitrophenyl amino acids corresponding to casein and preliminary study of the structure of β-casein). *Anales Fac. Quim. Farm., Univ. Chile*, 13 (1961) 11–20; *C.A.*, 57 (1962) 17025c.

KRAWCZYK, A.: Application of isatin to the fingerprint technique in the study of protein structure. *Bull. Acad. Polon. Sci., Ser. Sci. Biol.*, 10 (1962) 87–90; *C.A.*, 57 (1962) 14093e.

20. PROTEINS

IVANOVA, T. M. AND RUBIN, B. A.: (Codehydrase I oxidation by peroxidase). *Dokl. Akad. Nauk SSSR*, 150 (1963) 414–416.

LINSKENS, H. F. AND SCHRAUWEN, J. A. M.: α-Amylase-Nachweis auf dem Papierchromatogramm. *Naturwissenschaften*, 50 (1963) 333.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

HAINES, J. A., REESE, C. B. AND LORD TODD: The methylation of guanosine and related compounds with diazomethane. *J. Chem. Soc.*, (1962) 5281–5288.

JOHNSON, A. W. AND SHAW, N.: Some reactions of the vitamin B_{12} coenzyme. *J. Chem. Soc.*, (1962) 4608–4614.

KLÖCKING, H. P.: Über chemische Nachweise von Brevinarcon, Thiogenal, Thiotyr und Trapanal auf Papierchromatogrammen. *Pharmazie*, 18 (1963) 342–345.

KOBATA, A.: Isolation and identification of two novel uridine nucleotide oligosaccharide conjugates from human milk. *Biochem. Biophys. Res. Commun.*, 7 (1962) 346–350; *C.A.*, 57 (1962) 17033e.

LEDVINA, M. AND KÁCL, K.: (The detection of therapeutic doses of Sodanton in urine). *Acta Univ. Carolinae, Med. Suppl.*, No. 14 (1961) 317–322; *C.A.*, 57 (1962) 15739i.

RÜEGG, J. C. AND STRASSNER, E.: Sperrtonus and Nucleosidtriphosphate. *Z. Naturforsch.*, 18b (1963) 133–138.

VIDIC, E. AND GOEBEL, K.: (Renal excretion products of hypnotics). *Arch. Toxikol.*, 19 (1961) 85–111; *C.A.*, 57 (1962) 15740c.

22. ALKALOIDS

IIZUKA, K., YAMADA, M., SUZUKI, J., SEKI, I., AIDA, K., OKUDA, S., ASAI, T. AND TSUDA, K.: Microbiological hydroxylation of steroids. XVI. Conversion of morphine alkaloids with *Trametes sanguinea*. *Chem. Pharm. Bull. (Tokyo)*, 10 (1962) 67–70; *C.A.*, 57 (1962) 17201a.

WOJCIAK, Z.: (Chromatographic determination of alkaloids in extracts from animal materials by the use of cationitized paper). *Bull. Soc. Amis Sci. Lettres Poznan*, C11 (1962) 65–74; *C.A.*, 57 (1962) 16985g.

WOJCIAK, Z.: (Chromatographic quantitative determination of codeine, pilocarpine, and papaverine in extracts from animal materials (by use of cationitized paper).). *Bull. Soc. Amis Sci. Lettres Poznan*, C11 (1962) 75–80; *C.A.*, 57 (1962) 17002g.

ZARNACK, J. AND PFEIFER, S.: Versuche zur quantitativen papierchromatographischen Erfassung von Alkaloiden in Drogen. I. Mitt. Trennmethodik. *Pharmazie*, 18 (1963) 288–294 — the separation of opium alkaloids on buffered "segment strips".

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

KITTO, G. B. AND BRIGGS, M. H.: The biosynthetic pathway of nicotinamide adenine dinucleotide in an insect (*Hemideima thoracica*). *New Zealand J. Sci.*, 5 (1962) 237–240; *C.A.*, 57 (1962) 12889c.

MICHNIEWICZ, M.: (Chromatographic analysis of growth regulators from winter wheat plants treated with gibberellin). *Acta Agrobotan.* (*Warsaw*), 11 (1962) 197–211; *C.A.*, 57 (1962) 14217c.

MURACHI, T., TSUKADA, K. AND HAYAISHI, O.: Metabolic fate of kynurenic acid-C^{14} intraperitoneally administered to animals. *Biochemistry*, 2 (1963) 304–308.

OKANENKO, A. S., REINGARD, T. A., BERSHTEIN, B. I. AND OSTAPLYUK, A. N.: (The biochemical characteristics of healthy and diseased potatoes). *Biokhim. Plodov i Ovoshchei, Akad. Nauk SSSR, Inst. Biokhim.*, No. 7 (1962) 85–95; *C.A.*, 57 (1962) 12914c.

VOGEL, R., SCHIEVELBEIN, H., AND ECKERT, I.: (Histamine in hops and beer.) *Brauwissenschaft*, 15 (1962) 242–244; *C.A.*, 57 (1962) 13016f.

YAMANO, T., KOSUMOTO, M., YAMATODANI, S. AND ABE, M.: Ergot fungus. XLV. Production of indole-isopropionic acid and its esters by ergot fungi. *Takeda Kenkyusho Nempo*, 20 (1961) 33–41; *C.A.*, 58 (1963) 763g.

24. ORGANIC SULPHUR COMPOUNDS

BOYLAND, E. AND NERY, R.: Arylhydroxylamines. Part II. Phenylhydroxylamine-N- and -O-sulphonic acids. *J. Chem. Soc.*, (1962) 5217–5222 — R_F values of 12 phenylhydroxylamine derivatives.

DURO, F.: (Chromatographic identification of thiourea and thiouracil in fruit juices). *Boll. Sedute Accad. Gioenia Sci. Nat. Catania*, 6 (1961) 125–130; *C.A.*, 57 (1962) 17152d.

25. ORGANIC PHOSPHORUS COMPOUNDS

HAKOMORI, S. I., ISHIMODA, T. AND NAKAMURA, K.: Separation and characterisation of two sulfatides of brain. *J. Biochem.* (*Tokyo*), 52 (1962) 468–469.

KAUSS, H. AND KANDLER, O.: Adenosindiphosphatglucose aus *Chlorella*. *Z. Naturforsch.*, 17b (1962) 858–860.

LAMPRECHT, W. HEINZ., F., AND DIAMANTSTEIN, T.: (Phosphorylation of D-glyceric acid to 2-phospho-D-glyceric acid with glycerate kinase from liver. II. Identification of reaction products by paper chromatography). *Z. Physiol. Chem.*, 328 (1962) 204–206; *C.A.*, 57 (1962) 14169i.

LETTERS, R.: Chromatography of yeast lipids on silicic acid. *J. Inst. Brewing*, 68 (1962) 318–321; *C.A.*, 57 (1962) 15589b — paper and thin-layer chromatography.

26. METALLO-ORGANIC COMPOUNDS

WAGNER, G. AND NUHN, P.: Über O,Se- und N,Se-Bis-glykoside von 4-Hydroxy- bzw. 4-Aminoselenophenol. *Arch. Pharm.*, 296 (1963) 374–383 — R_F values of 17 glycosides.

27. VITAMINS

GALE, P. H., ARISON, B. H., TRENNER, N. R., PAGE, Jr., A. C. AND FOLKERS, K.: Coenzym Q. XXXVI. Isolation and characterization of coenzyme Q_{10} (H-10). *Biochemistry*, 2 (1963) 196–199.

GALE, P. H., ARISON, B. H., TRENNER, N. R., PAGE, Jr., A. C. AND FOLKERS, K.: Characterization of vitamin K_9 (H) from *Mycobacterium phlei*. *Biochemistry*, 2 (1963) 200–203.

MARTIN, J. L. AND SHAW, W. H. C.: Hydroxocobalamin: its examination and determination in parenteral injection solution by paper chromatography. *Analyst*, 88 (1963) 292–296.

REMBOLD, H. AND BUSCHMANN, L.: Struktur und Synthese des Neopterins. *Chem. Ber.*, 96 (1963) 1406–1410.

TRAVIS, J. AND ROBINSON, A. D.: The separation and estimation of riboflavine and its derivatives in mixtures. *Can. J. Biochem. Physiol.*, 40 (1962) 1251–1252; *C.A.*, 57 (1962) 15438e.

28. ANTIBIOTICS

OCHAB, S., MALYSZ, D. AND BOROWIECKA, B.: (The determination of erythromycin in the presence of tetracycline). *Chem. Anal.* (*Warsaw*), 8 (1963) 597–600.

29. INSECTICIDES AND OTHER PESTICIDES

BLUM, M. S. AND CALLAHAN, P. S.: Chemical and biological properties of the venom of the imported fire ant and the isolation of the insecticidal component. *Intern. Congr. Entomol., Proc., 11th, Vienna,* No. 3 (1960) 290–293; *C.A.*, 57 (1962) 17224a.

DEDEK, W., GRIMMER, F. AND KOCH, H.: (Radioactive labeled esters of H_3PO_4. I. Preparation of ^{32}P labelled β-methylthioethyl dimethyl thiophosphate isomers and their hydrolytic decomposition in plants). *Isotopen Techn.*, 2, No. 5 (1962) 150–157; *C.A.*, 57 (1962) 12953h.

VAINTRAUB, F. P.: (Determination of microquantities of DDT in biological materials by using the Schechter and Haller method with complementary chromatographic identification). *Vopr. Pitaniya*, 21 (1962) 55–60; *C.A.*, 57 (1962) 15439i.

30. SYNTHETIC AND NATURAL DYES

BROWNLEY, CH. A., Jr. AND LACHMAN L.: Preliminary report on the comparative stability of certified colorants with lactose in aqueous solution. *J. Pharm. Sci.*, 52 (1963) 86–93.

FISCHER, F., KAPITZA, W., GERSCH, M. AND UNGER, H.: Sind die Neurohormone der Arthropoden identisch mit fluoreszierenden Substanzen aus dem Nervensystem? *Z. Naturforsch.*, 17b (1962) 834–836.

KÜHN, A.: Die zeitliche Folge der Bildung der Ommochrome in den Imaginalaugen von *Ephestia* und *Ptychopoda*. *Z. Naturforsch.*, 18b (1963) 252–254.

NAGAI, S.: Production of respiration-deficient mutants of yeast by some quinoneimine dyes. *Exptl. Cell Res.*, 27 (1962) 14–18; *C.A.*, 57 (1962) 15613c.

SACCHETTA, R. A. AND SIMON, G.: (Horizontal radial chromatography of red pepper on paper disks. Analytical application. *Rev. Asoc. Bioquim. Arg.*, 26 (1961) 124–131; *C.A.*, 57 (1962) 15439f.

ŠESTÁK, Z.: (The application of paper chromatography of chlorophylls and carotenoids to the algology). *Preslia*, 35 (1963) 123–134.

32. PHARMACEUTICAL APPLICATIONS

GSTIRNER, F. AND BRAUN, W.: Über Inhaltsstoffe der weissen koreanischen Ginseng-Droge. *Arch. Pharm.*, 296 (1963) 384–389.

HAVERLAND, F.: *Polygonum aviculare* L., Der Vogelknöterich. *Pharmazie*, 18 (1963) 59–87.

KRITIKOS, P. G. AND PHILIANOS, S. M.: Contribution à la pharmacognosie des hellébores (*Helleborus cyclophyllus* Boiss.). *Pharm. Acta Helv.*, 38 (1963) 224–234.

SATO, K.: Identification of nonbarbiturate sedatives in urine. *Kagaku Keisatsu Kenkyusho Hokoku*, 15 (1962) 237–243; *C.A.*, 57 (1962) 16986d.

TUNMANN, P. AND STIERSTORFER, N.: Zur Kenntnis der Inhaltsstoffe aus der Wurzel von *Harpagophytum procumbens* DC. 2. Mitt. Über das Vorkommen von Stachyose und über ein weiteres Glukosid. *Deut. Apotheker-Ztg.*, 103 (1963) 395–397.

33. INORGANIC SUBSTANCES

BASOLO, F., LEDERER, M., OSSICINI, L. AND STEPHEN, K. H.: A paper chromatographic study of some platinum(II) compounds. II. The separation of *cis*- and *trans*-dihalogenodiammine-platinum(II) complexes. *J. Chromatog.*, 10 (1963) 262–267.

34. RADIOACTIVE COMPOUNDS

DOMAN, N. G., ROMANOVA, A. K. AND TERENT'EVA, Z. A.: (C^{14} assimilation products in the *Rhodopseudomonas issatchenkoi* bacteria). *Mikrobiologia*, 31 (1962) 193–198; *C.A.*, 57 (1962) 13000i.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

BOBBITT, J. S.: *Thin Layer Chromatography*, Reinhold Co., New York, 1963, 250 pp.

3. TECHNIQUES I

DAVIES, B. H.: A simple saturation chamber for thin-layer chromatography. *J. Chromatog.*, 10 (1963) 518–521.

PATAKI, G. AND KELEMEN, J.: Einfluss der Schichtbereitungsweise auf die Reproduzierbarkeit der R_F in der Dünnschichtchromatographie. *J. Chromatog.*, 11 (1963) 50–54.

RANDERATH, E. AND RANDERATH, K.: Polyphosphat-Cellulose, ein neuer Kationenaustauscher. Herstellung und Anwendung in der Dünnschicht Chromatographie. *J. Chromatog.*, 10 (1963) 509–510 — nucleic bases and nucleosides.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

BADGER, G. M., DONNELLY, J. K. AND SPOTSWOOD, T. M.: Thin-layer chromatography using partially acetylated cellulose as adsorbent. *J. Chromatog.*, 10 (1963) 397 — R_F values of 7 hydrocarbons.

KUCHARCZYK, N., FOHL, J. AND VYMĚTAL, J.: Dünnschichtchromatographie von aromatischen Kohlenwasserstoffen und einigen heterocyclischen Verbindungen. *J. Chromatog.*, 11 (1963) 55–61 — R_F values of 30 substances.

6. ALCOHOLS

DUMAZERT, CH., GHIGLIONE, C. AND PUGNET, T.: Séparation et identification d'esters acétiques de polyols et de glucides par chromatographie en "couches minces". *Bull. Soc. Chim. France*, (1963) 475–476.

9. OXO COMPOUNDS

SIEPER, H., LONGO, R. AND KORTE, F.: Zur chemischen Klassifizierung von Pflanzen. XXIII. Trennung und Bestimmung pflanzlicher Polyhydroxyanthrachinone durch Dünnschicht-Chromatographie. *Arch. Pharm.*, 296 (1963) 403–411 — R_F values of 6 anthraquinones.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

PASTUSKA, G. AND PETROWITZ, H. J.: Dünnschicht-Chromatographie *cis-trans* isomerer Carbonsäuren. *J. Chromatog.*, 10 (1963) 517 — R_F values of 10 acids.

14. STEROID GLYCOSIDES

FAUCONNET, L. AND WALDESBÜHL, M.: Analyse des cardénolides digitaliques par chromatographie sur couche mince. *Pharm. Acta Helv.*, 38 (1963) 423–429 — R_F values of 21 glycosides and aglycones.

15. TERPENE DERIVATIVES

TYIHÁK, E., VÁGUJFALVI, D. AND HÁGONY, P. L.: Die gas- und dünnschichtchromatographische Trennung der stereoisomeren Farnesole und ihrer Derivate. *J. Chromatog.*, 11 (1963) 45–49 — chromatography of 16 substances.

18. AMINO ACIDS

RATNEY, R. S.: A method for diazotising (2,4-dinitro-5-aminophenyl)-amino acids on thin-layer chromatograms. *J. Chromatog.*, 11 (1963) 111–112.

22. ALKALOIDS

CONE, N. J., MILLER, R. AND NEUSS, N.: Alkaloids of *Vinca rosea* Linn. (*Catharanthus roseus* G. Don). XV. Analysis of *Vinca* alkaloids by thin-layer chromatography. *J. Pharm. Sci.*, 52 (1963) 688–692 — chromatography of 26 alkaloids in 11 systems.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

OKUI, S., SUZUKI, Y., MOMOSE, K. AND OGAMO, A.,: Chemical properties and enzymatic reduction of neotetrazolium chloride. *J. Biochem. (Tokyo)*, 53 (1963) 500–502.

24. ORGANIC SULPHUR COMPOUNDS

KHO, B. T. AND SHELDON KLEIN: Thin-layer chromatography in drug analysis. II. Procedure for the identification of various N^4-substituted sulfonamides. *J. Pharm. Sci.*, 52 (1963) 404–406 — 10 sulphonamides.

25. ORGANIC PHOSPHORUS COMPOUNDS

CLEMENT, J. AND DI CONSTANZO, G.: Séparation chromatographique, identification et analyse des phospholipides de la muqueuse intestinale du rat à jeun. *Bull. Soc. Chim. Biol.*, 45 (1963) 127–136.

32. PHARMACEUTICAL APPLICATIONS

BÖHME, H. AND KREUTZIG, L.: Zur Papier- und Dünnschichtchromatographie von Aloe-Drogen. *Deut. Apotheker-Ztg.*, 103 (1963) 505–508.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

GEHRMANN, J. AND SCHNEIDER, F. L.: Thin-layer chromatography in analysis of photometric reagents. *Microchem. J.*, 6 (1962) 561–562.

Gas Chromatography

1. REVIEWS AND BOOKS

ACKMAN, R. G. AND VANDENHEUVEL, F. A.: Preparative gas–liquid chromatography. *Chem. Can.*, 15, No. 2 (1963) 34–36 — a review with 67 references.

GLUECKAUF, E.: Separation of isotopes by gas chromatography and ion-exchange separation. *J. Chem. Phys.*, 60 (1963) 73–80 — a critical review.

HOWARD, G. E.: An introduction to the principles and technique of gas chromatography. *Chem. Ind. (London)*, (1963) 622–628.

TEISSEIRE, P.: (Use of gas chromatography in perfume chemistry). *Bull. Soc. Chim. France*, (1963) 384–388.

VIGDER'GAUZ, M. S., AFANAS'EV, M. I. AND GOL'BERT, K. A.: (Trace analysis by means of gas chromatography). *Usp. Khim.*, 32 (1963) 754–771 — methods tabulated according to the type of compound; 91 references; incomplete.

WALDEYER-HARTZ, G. VON: (New apparative development in gas chromatography. Part II). In ANGELÉ H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 252–269 — a review of GC instrumentation 1961–1963; 51 references.

WURST, M.: (Gas Chromatography. II. Methodics of quantitative analysis). *Chem. Listy*, 57 (1963) 615–628 — 24 references.

2. FUNDAMENTALS, THEORY AND GENERAL

2a. Gas–liquid systems

MCQUARRIE, D. A.: On the scholastic theory of chromatography. *J. Chem. Phys.*, 38 (1963) 437–445 — solution as a Poisson's process.

2b. Gas–solid systems

TOTH, J.: (Evaluation of adsorptive fillings applied in gas–solid chromatography on basis of a new theory of adsorption). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 200–213 — expressed net adsorption energy and surface inhomogeneity.

2c. Thermodynamics and theoretical relationships

FRANC, J. AND MICHAJLOVA, S.: (Identification of organic substances by means of gas chromatography through chromatographic spectra). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 32–47 — identification from 4 retention values and ΔR.

MAGEE, E. M.: The course of a reaction in a chromatographic column. *Ind. Eng. Chem., Fundamentals*, 2, No. 1 (1963) 32–36 — definition of partial differential equations.
ROSE, A. AND SCHRODT, V. N.: Correlation and prediction of vapor pressures of homologs. Use of structure parameters and gas chromatography data. *J. Chem. Eng. Data*, 8 (1963) 9–13 — substitution of relative volumes in the vapour pressure equation.

2d. General

AGENO, M. AND FRONTALI, C.: Analysis of frequency distribution curves in overlapping Gaussians. *Nature*, 198 (1963) 1294–1295 — equations for approximate evaluation; applicable in GC.
ARAKI, S. AND KATO, T.: Peak distortion in gas chromatograms. *Japan Analyst*, 12 (1963) 179–181.
JONES, C. E. R. AND KINSLER, D.: (A statistic evaluation of gas partition chromatography as a method for quantitative analysis). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 85–99 — for components of 0.25–64 % content in mixtures; average errors were 0.17 % for two- and 0.20 % for six-component systems.
WIGDER'GAUS, M. S. AND GOL'BERT, K. A.: (Scheme of gas chromatographic separation of component-rich mixtures). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors), *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 270–298 — theory of multi-column systems and partially and fully back flushing.

3. TECHNIQUES I

3a. Detectors

NOVÁK, J. AND JANÁK, J.: (Pneumatic analog of Wheatstone's bridge as detector of pneumatic stream effects of gas-chromatographic fractions). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 100–108 — theory and construction details are given; sensitivity as for katharometers.
PRÖSCH, U. AND ZÖPFL, H. J.: (Testing of a gas chromatograph for a two-detector system). In *Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 140–149 — katharometer with β-ray ionization detector.

3b. Column performance and filling studies

LIGUZOVA, L. I. AND VITUKHINA, A. S.: (Preparation of sorbent CaX (10X), and some of its catalytic properties). *Khim. i Tekhnol. Topliv. Masel*, No. 6 (1963) 17–21 — separation of permanent gases and ethane.
ROGERS, L. B. AND ALTENAU, A. G.: New adsorbents for gas chromatography. *Anal. Chem.*, 35 (1963) 915–916 — Cu-complexes (Py, NH_3), $CdSO_4$ and $MgCl_2 \cdot 2H_2O$ of porous structure separate hydrocarbons and oxygenated compounds at 38–130°.
URONE, P. AND KATNIK, R. J.: A new and efficient liquid phase for gas liquid chromatography of oxygenated substances. *Anal. Chem.*, 35 (1963) 767–768 — nonylphenol; retention volumes of 14 oxygenated hydrocarbons.
URONE, P. AND PECSOK, R. L.: Gas chromatographic behavior of C_1–C_4 saturated alcohols and water on polyethylene glycol substrate. Effect of solid support treatment. *Anal. Chem.*, 35 (1963) 837–841 — relative retention volumes and activity coefficients are largely independent of the treatment of solid support at 20 % wt. coating; retention data at 70°.

3c. Apparatus, accessories and materials for GC

COGLIANO, J. A.: Apparatus for rapid pyrolysis under controlled conditions. *J. Sci. Instr.*, 34 (1963) 439–440 — detailed wiring diagram; also applicable in GC.
COOMBS, W. F.: A versatile new recorder. *Research/Development*, 14 (1963) 36–38.
FEJES, P., ENGELHARDT, J. AND SCHAY, G.: (A new sampling device for capillary chromatography). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors), *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 21–31 — construction details.
LOWRY, R. R.: A solid sample injector system for gas chromatography. *18th Annual Northwest Regional Meeting Am. Chem. Soc.*, Bellingham, Wash., June 17–18, 1963.
NATELSON, S. AND STELLATE, R. L.: Apparatus for extraction of gases for injection into the gas chromatograph. Application to oxygen and nitrogen in jet fuel and blood. *Anal. Chem.*, 35 (1963) 847–851 — design of extractor and injector; 0.2 ml samples.
OBST, D.: (Specialities in construction of gas chromatograph GCI 2). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 109–123 — katharometer; temperature to 400°.
PETZELT, B.: (Review on developments of Czechoslovak laboratory gas chromatography equipment). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 124–139 — Chrom. 1: temperature to 350°, flame ionization; Chrom. 2: combined packed and capillary columns, integrator; Chrom. 3: combined detectors, katharometer, argon and flame ionization.

VANGO, S. P.: Gas handling apparatus for gas chromatography. *Chemist-Analyst*, 52 (1963) 53–54.
VANKO, A., BERIAC, L., ADÁSEK, Š. AND HANKUS, A.: (Automatic plant chromatograph). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 214–224 — all design details; thermostat 50°; sampling errors 0.5 vol. %.

4. TECHNIQUES II

4a. Preparative-scale GC

SAKODYNSKIĬ, K. I., VOLKOV, S. A., BRASHNIKOV, V. V. AND SHAVORONKOV, N. V.: (Separation on preparative columns). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 164–178 — specific loading 1.4–11 g/cm^2; columns of 13–30 mm I.D.

4d. Special microtechniques

BENEDECK, P. AND MÜLLER, L.: (Vapour pressure determination by means of gas chromatography). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 1–8 — relative errors below 5%.
EDGECOMBE, F. H. C.: Copolymerization by vapour phase chromatography. *Nature*, 198 (1963) 1085–1086 — automatic removal of sample from catalytic reaction products for GC analysis.
GARZÓ, G. AND SZÉKELY, T.: (Investigation of thermal processes in space-linked silico-organic polymers by means of reaction gas chromatography). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 48–70 — pyrolytic techniques.
PARSONS, M. L., PENNINGTON, S. N. AND WALKER, J. M.: A rapid combustion method for nitrogen determination utilizing gas chromatography. *Anal. Chem.*, 35 (1963) 842–844 — high frequency induction combustion; errors 0.1% abs. for 1–10 mg samples of organic compounds and 10–20 mg of blood.

4e. Automation

NEBLETT, J. B. AND MEARS, F. C.: In-line chromatograph teams up with computer for better process control. *Oil Gas J.*, 60, No. 1 (1963) 94–96 — a brief description.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5b. Hydrocarbons

DÖRING, C. E. AND HAUTHAL, H. G.: (Gas chromatographic investigation of C_6-hydrocarbons). *Acta Chim. Hung.*, 37 (1963) 125–135 — retention data of 56 hydrocarbons on 9 stationary phases at 25–50°.
KAUFMANN, H., WELKER, J. AND BÄTZ, R.: (Hydro-refining of pyrolytic gasoline from trapped oil originated from olefin pyrolysis units for the production of benzene). *Acta Chim. Hung.*, 36 (1963) 131–144 — GC of C_6–C_8 aromatics on Aroxan C at 80°.
SHOPOV, D., KOCEV, N. AND GEORGIEVA, K.: (Identification of C_7–C_9 aromatic hydrocarbons from catalytic gasoline by means of gas–liquid chromatography). *Acta Chim. Hung.*, 37 (1963) 137–145 — retention volumes on fluorene picrate.
SZYNAGEL, P.: (Gas chromatographic determination of individual hydrocarbons in gasoline from Polish and Romashkino crudes and their separation). *Acta Chim. Hung.*, 37 (1963) 227–238 — GC on squalane + quinoline, diisobutyl phthalate, *n*-hexadecane.
WINGERTER, K. H.: (C_9-Aromatics in the reformate of a gasoline from Romashkino crude). *Acta Chim. Hung.*, 37 (1963) 177–189.

5c. Halogen derivatives of hydrocarbons

GREENE, S. A. AND WACHI, F. M.: Separation of some low molecular weight fluorocarbons by gas chromatography. *Anal. Chem.*, 35 (1963) 928–929 — 12 fluorocarbons to C_4F_8; GLC on $CH_2{:}CH{\cdot}COO{\cdot}CH_2(CF_2CF_2)_3$ at 0°, GSC on silica gel with temperature programming.
HEITZMAN, R. J., PATRICK, C. R., STEPHENS, R. AND TATLOW, J. C.: Fluorocyclopentanes. Part I. The 1H-, 2H- and 1H, 3H-octafluorocyclopentanes and 1H, 3H/2H-heptafluorocyclopentane. *J. Chem. Soc.*, (1963) 281–289 — retention data on dinonyl phthalate at 60–100°.
HYNES, J. B., BISHOP, B. C., BANDYOPADHYAY, P. AND BIGELOW, L. A.: The action of elementary fluorine upon organic compounds. XXVI. The direct fluorination of some perfluoroalkyls-*s*-triazines. *J. Am. Chem. Soc.*, 85 (1963) 83–86 — fluorocarbons on Kel-F (—80° to 40°), hexadecane (40–80°), dioctyl phthalate (80–120°).
MAZOŃSKI, T., KULICKI, Z. AND PINDUR, B.: (The alkylation of chlorobenzene by isopropyl alcohol in the presence of various catalysts. I. The alkylation in the presence of anhydrous aluminium chloride). *Roczniki Chem.*, 37 (1963) 569–573 — isomers of isopropylchlorobenzene on polyoxymethylene sorbitol monostearate at 150°.

6. ALCOHOLS

ARCUS, C. L., PAGE, J. M. J. AND REID, J. A.: Olefinic addition with asymmetric reactants. Part VII. The hydrogenation of (±)-3-methylenepentan-2-ol. *J. Chem. Soc.*, (1963) 1213–1216 — retention data of diastereoisomers on PEG 400 at 75°.

JAFFE, F., STEADMAN, T. R. AND MCKINNEY, R. W.: Primary products of decalin autooxidation. *J. Am. Chem. Soc.*, 85 (1963) 351–353 — retention volumes of decanols on Ucon LB-550 at 160°.

MAUREL, A.: (Application of gas chromatography to the analysis of alcohols). *Bull. Soc. Chim. France*, (1963) 316–319.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

CARTER, H .E. AND HENDRICKSON, H. S.: Biochemistry of the sphingolipids. Part XV. Structure of phytosphingosine and dehydrophytosphingosine. *Biochemistry*, 2 (1963) 389–393 — retention data of aldehydes and fatty acid esters on DEG-glutarate and QF-1 at 180°; combination with TLC.

CASON, J. AND MILLER, W. T.: Complexity of the mixture of fatty acids from tubercle bacillus. C_{16} and C_{17} acids from virulent strains. *J. Biol. Chem.*, 238 (1963) 883–887 — retention data of fatty acid methyl esters, aldehydes and ketones on silicone grease and DEGS at 142–200°.

FUNASAKA, W., KOJIMA, T. AND KIMOTO, J.: Gas-chromatographic analysis of mixtures of benzenepolycarboxylic acids. *Japan Analyst*, 12 (1963) 247–252.

KATES, M., CHAN, T. H. AND STANACEV, N. Z.: Aliphatic diether analogs of glyceride-derived lipids. Part I. Synthesis of D-α,β-dialkyl glyceryl ethers. *Biochemistry*, 2 (1963) 394–397 — retention data of ethers and esters on SE-30 at 220°.

KOMERS, R.: (Investigation on gas-chromatographic separation of benzenemono- and dicarboxylic acid methyl esters). *Collection Czech. Chem. Commun.*, 28 (1963) 1549–1556 — retention volumes on 6 stationary phases: optimum 43 % tetrakis-O-(2-cyanoethyl)-pentaerythritol + 57 % PEGA at 176°.

MEINWALD, J., CABANA, S. S. AND CHADNA, M. S.: Peracid reactions. III. The oxidation of bicyclo[2.2.1]-heptadiene. *J. Am. Chem. Soc.*, 85 (1963) 582–585 — retention volumes of carboxylates at 160°.

PREVOT, A.: (Application of gas chromatography to the analysis of lipids). *Bull. Soc. Chim. France*, (1963) 313–316.

RIDDLE, V. M.: Determination of traces of organic acids in small samples. Gas liquid chromatography of the total condensate. *Anal. Chem.*, 35 (1963) 853–859 — C_3–C_8 in p.p.b. quantities are isolated by steam distillation and analysed by GC in 0.5–100 μg range.

VASILESCU, V.: (Contribution to the explanation of processes for selective high-pressure reduction of the carboxyl group of unsaturated fatty acids. Gas chromatography of degradation products of ozonides originated from unsaturated fatty alcohols). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 225–251 — fatty acid esters at 250°; interesting structure study.

ZEMAN, I.: (Gas chromatography of fatty acids with triple bonds). In ANGELÉ, H. P. AND STRUPPE. H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 332–338 — retention volumes of C_{18}–C_{22} acids on Apiezon L at 220° and on PEGA at 180°.

12. ORGANIC PEROXIDES

BUKATA, S. W., ZABROCKI, L. L. AND MCLAUGHLIN, M. F.: Gas chromatography of organic peroxides. *Anal. Chem.*, 35 (1963) 885–886 — retention times of 8 peroxides on didecyl phthalate and silicone grease at 70–140°.

13. STEROIDS

KIRSCHNER, M. A. AND LIPSETT, M. B.: Gas–liquid chromatography in the quantitative analysis of urinary 11-desoxy-17-keto-steroids. *J. Clin. Endocrinol. Metab.*, 23 (1963) 255–260 — retention data of some steroids on QF-1-0065 at 187°.

WECHTER, W. J. AND MURRAY, H. C.: 11-Oxygenated pregnenolones. II. Synthesis of 11α-hydroxy-, 11β-hydroxy-, and 11-keto-pregnenolenes and 11-keto-17α-pregnenolone. *J. Org. Chem.*, 28 (1963) 755–670 — retention volumes of esters and diols on SE-30.

15. TERPENE DERIVATIVES

BLAKE, S. AND JONES, G.: Extractives from *Eperna Falcata*. The petrol-soluble constituents. *J. Chem. Soc.*, (1963) 430–433 — retention data for methyl esters of diterpene acids on SE-30 at 175°.

HAMER, J. C.: Analysis of seed oil of six tropical plant species by gas chromatography. *Diss. Abstr.*, 23 (1963) 2322.

KATAGUE, D. B. AND KIRCH, E. R.: Analysis of the volatile components of Ylang-ylang oil by gas chromatography (terpene constituents). *J. Pharm. Sci.*, 52 (1963) 252–258 — on Ucon 50 at 160–190°.

MORYASHCHEV, A. K.: (Determination of the composition of certain ethereal oils by gas chromatography). *Zh. Analit. Khim.*, 18 (1963) 401–405.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

ARAD-TALMI, Y., LEVY, M. AND VOFSI, D.: Gas chromatographic analysis of aliphatic nitriles in aqueous acidic solution. *J. Chromatog.*, 10 (1963) 417–420 — C_3, C_4 and C_6 mono- and dinitriles extracted with *o*-dichlorobenzene and dried over Na_2SO_4, are separated on LAC-2-R-466 + H_3PO_4 at 70° (for mono-), at 220° (for dibasic nitriles).

PIERCE, A. C. AND JOULLIÉ, M. M.: Action of primary and secondary amines on trihalogenated esters. *J. Org. Chem.*, 28 (1963) 658–662 — retention volumes of esters, amides and carbamates on silicone oil at 195–245°.

18. AMINO ACIDS

RÜHLMANN, K. AND MICHAEL, G.: (Gas chromatography of N-trimethyl-silyl amino acids trimethyl-silyl esters). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 150–163 – semiquantitative results for some acids on silicone oil OE 4018/20000 at 160° and 200°.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

BOOTH, H., JOHNSON, A. W., JOHNSON, F. AND LANGDALE-SMITH, R. A.: Methylation of some pyrroles and 2-pyrrolines. *J. Chem. Soc.*, (1963) 650–661 — retention data on silicone oil at 152° and 167°.

24. ORGANIC SULPHUR COMPOUNDS

SETZKORN, E. AND CAREL, A. B.: Analysis of alkyl aryl sulphonates by micro-scale desulphonation and gas chromatography. *J. Am. Oil Chemists' Soc.*, 40 (1963) 57–59.

25. ORGANIC PHOSPHORUS COMPOUNDS

FEINLAND, R., SASS, J. AND BUCKLER, S. A.: Determination of trialkyl phosphines and their oxidation products by gas liquid chromatography. *Anal. Chem.*, 35 (1953) 920–921 — relative retention times of 8 compounds on silicone grease 200 at 240° and on Reoplex 400 at 206°.

26. METALLO-ORGANIC COMPOUNDS

ECKNIG, W., ROTZSCHE, H. AND KRIEGSMANN, H.: (Separation and identification of hexamethylcyclotetrasiloxane isomers by means of a combination of analytical gas chromatography with I.R.-spectroscopy). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 9–20 — complete separation on squalane at 106°.

GARZÓ, G. AND TILL, F.: Argon ionization detector in gas–liquid chromatography of organosilicone compounds. *Talanta*, 10 (1963) 583–589 — retention volumes on silicone grease and Apiezon M at 75–100°; pyrolysis chamber.

LENGYEL, B., GARZÓ, G. AND SZÉKELY, T.: (Some problems concerning the gas-chromatographic analysis of methylchlorosilanes). *Acta Chim. Hung.*, 37 (1963) 37–51 — complete separation on nitrobenzene.

PHILLIPS, C. S. G., POWELL, P. AND SEMLYN, J. A.: The chromatography of gases and vapours. Part VII. Some substituted borazoles. *J. Chem. Soc.*, (1963) 1202–1207 — retention volumes on squalane and Carbowax 4000 at 100°.

WURST, M.: (Analysis of linear and cyclic polydimethylsiloxanes by means of gas chromatography). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 318–331 — retention volumes of D_3–D_8 and M_2–M_2D_5 at 150–195°; Green's detector.

29. INSECTICIDES AND OTHER PESTICIDES

BOSIN, W. A.: Analysis of pesticides residue using microcoulometric temperature-programmed gas chromatography. *Anal. Chem.*, 35 (1963) 833–837 — detailed designs: retention times of 42 Cl- and S-pesticides; temperature programming to 240°.

31. PLASTICS AND THEIR INTERMEDIATES

SZEPESY, L. AND SIMON, J.: (Gas-chromatographic investigation of impurities of methyl methacrylate). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors). *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 190–199 — on polypropylene glycol + CaC_2 at 150°.

TWEET, O. AND MILLER, W. K.: Determination of residual monomer in polymer emulsions by rapid distillation and gas chromatography. *Anal. Chem.*, 35 (1963) 852–853 — up to 4 p.p.m. of ethyl acrylate in 50 g sample with toluene as internal standard.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

FUJITA, K. AND KWAN, T.: Quantitative determination of para- and ortho-hydrogen, hydrogen deuteride and deuterium by gas chromatography. *Japan Analyst*, 12 (1963) 15–20.

HAUBACH, W. J. AND WHITE, D.: Chromatographic separation of the isotopic and nuclear-spin species of the hydrogens at low temperatures. *J. Chem. Phys.*, 60 (1963) 97–99.

ROFF, J. A.: Application of gas chromatography to vacuum-fusion analyses. *Chem. Ind. (London)*, (1963) 537–538 — molecular sieve 5A at 95°; negative Lovelock detector technique.

33b. Volatile inorganic compounds

GALWEY, A. K.: Use of the argon gas chromatograph in the determination of carbon in steel. *Talanta*, 10 (1963) 310–314 — errors ± 0.01 % of C in 0.1–2 g steel samples with 0–0.3 % C, determined as CO_2.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

ARCHER, D. A. AND BOOTH, H.: The thermal decomposition of quaternary ammonium hydroxides. Part I. Methohydroxides derived from N-dialkylanilines and related compounds. *J. Chem. Soc.*, (1963) 322–330 — retention data on Apiezon at 193°; substituted anilines, amines and olefins.

COCKER, W. AND SHAW, S. J.: Extractives from woods. Part III. Extractives from *Manilkara Bidentata*. *J. Chem. Soc.*, 1 (1963) 677–680 — retention volumes of hydrocarbons at 205° and fatty acid methyl esters at 195° on SE-30 and DEGS.

GATRELL, R. L.: A mixed-substrate column for gas chromatographic analysis of lacquer thinners. *Anal. Chem.*, 35 (1963) 923–924 — retention times of 20 solvents at 108°; temperature programming for routine analyses.

GORLAS, J.: (Gas chromatography in waste water analysis). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, Akademie-Verlag, Berlin, 1963, pp. 71–84 — aromatics and phenols after extraction (in mg/l), details for continuous extracting unit.

HISHTA, C. AND BOMSTEIN, J.: Gas chromatography of polar compounds of low volatility. *Anal. Chem.*, 35 (1963) 924–927 — benzoquinolines, benzylphenols, phenyltoloxamines, *e.g.* up to mol. wt. 672 at 200–290°.

ISSENBERG, P. AND WICK, E. L.: Volatile components of bananas. *J. Agr. Food Chem.*, 11 (1963) 2–8 — retention volumes on UCON 50HB 2000 at 32–36° and 85°.

McKEE, H. C., RHOADES, J. W., WHEELER, R. J. AND BURCHFIELD, H. P.: Gas chromatographic measurement of trace contaminants in a simulated space cabin, *NASA (Natl. Aeron. Space Admin.), Tech. Note*, TN D-1825, March, 1963, 39 pp. — aldehydes, ketones, alcohols, freons and other compounds on Carbowax 1540 and PEG E 300 at 40°.

STIMSON, V, R., AND WATSON, E. J.: Catalysis by hydrogen halides in the gas phase. Part VIII. Methyl *tert.*-butyl ether and hydrogen bromide. *J. Chem. Soc.*, (1963) 524–527 — retention times at 72°.

WIGDER'GAUS, M. S. AND GOL'BERT, K. A.: (Gas chromatographic analysis of petrochemical products). In ANGELÉ, H. P. AND STRUPPE, H. G. (Editors): *Preprintbook Gas-Chromatographie 1963*, pp. 299–317 — summary of authors' own papers; 12 references.

A QUANTITATIVE APPROACH TO UNIDIMENSIONAL MULTIPLE CHROMATOGRAPHY*

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One very successful but infrequently-used technique for the resolution of closely related compounds is unidimensional multiple chromatography (UMC)[1-3], (any procedure involving the repeated irrigation of chromatographic supports in one direction). The excessive time consumed by repeated irrigations using this technique, has largely been circumvented by the speed of thin-layer chromatography (TLC)[4,5] and centrifugally accelerated chromatography[6]. UMC has some desirable features which make it an extremely useful analytical tool. A number of advantages are readily evident when UMC is used in conjunction with TLC or "open column chromatography". For example, time consuming labors are eliminated such as packing columns, extruding and streaking for band detection or monitoring large volumes of eluate or the contents of a large number of fractions, etc. Furthermore, no special or expensive apparatus is required for UMC, the traditional and simple equipment employed for ascending chromatography being adequate. While continuous chromatography (solvents allowed to drip from support) can theoretically afford greater resolution than UMC[3], the latter method remains a practical technique because of its ease of operation. Thus, the advantages of UMC described above will generally more than compensate for the greater resolving efficiency of continuous chromatography.

In a recent communication[3], the theory of UMC was developed and its practical and theoretical potentialities and the limitations discussed. In that article, a theoretical method for the selection of solvent systems which might separate closely related compounds was suggested, but the excessive labor involved in accumulating the relevant data made the approach founded in theory impractical. On that account, a simple empirical procedure based upon a few guiding rules was proposed for the selection of an appropriate chromatographic solvent. To aid in placing these guides on a more quantitative basis, tables have been compiled which relate the R_F values of two compounds to the number of solvent passes required for resolution by a preselected increment. Thus if the R_F values of two compounds are known or can be estimated, it will be possible, by consultation of Tables I–IV, to ascertain if the solutes can be resolved with the test solvent and when separation is possible, how many solvent excursions will be required.

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When the detection of an elongated spot on a chromatogram leads to the suspicion of the presence of two or more solutes, the normal procedure is to search for another solvent mixture which will resolve the spot into two bands. UMC is an alternate approach to the problem which is more attractive because it is more certain of success and may be less time consuming than extensive experimentation with other solvents. The rationale behind the use of UMC is that very similar compounds which are partially separated on one solvent pass, in most instances, can be completely resolved by further solvent irrigations. On the other hand, there is never any guarantee that replacing one solvent by another will ever lead to complete or even increased resolution of closely related solutes[3]. As an example, a survey of the literature and our own experience with several solvents failed to reveal a single solvent system (of 16 tested) which was capable of resolving fructose and arabinose on a single pass. However, the solutes are readily separated by UMC. The use of Tables I–IV for selecting a suitable solvent for the resolution of the two closely migrating saccharides is illustrated below.

EXPERIMENTAL

Computations

The following equation[1–3] relates the true or single pass R_F values of two compounds to their relative separation Δp after p passes:

$$\Delta p = (1 - R_{Fa})^p - (1 - R_{Fb})^p \tag{1}$$

where R_{Fa} and R_{Fb} are the R_F values of compounds A and B, respectively. Equation (1) was found to be more useful for computational purposes when rearranged into the form of eqn. (2):

$$\Delta p - [(1 - R_{Fa})^p - (1 - R_{Fb})^p] \geq 0\,. \tag{2}$$

The values of p in Tables I–IV were determined on the Indiana University IBM 650 computer programmed to select the smallest integral values of p, $p_{\min}$, which produced solution of eqn. (2) (provided a solution of the equation was possible). The value of $p_{\min}$ was found by incrementing p by unity until the condition demanded by eqn. (2) for a given set of experimental conditions was satisfied. Various experimental conditions were simulated by altering the values assigned Δp, R_{Fa} and R_{Fb}. After solution of eqn. (2) for the initial conditions, the values of Δp, R_{Fa} and R_{Fb} were then appropriately incremented until all useful combinations were considered.

Multiple chromatography

To demonstrate the use of Tables I–IV and illustrate the principles involved in UMC, sample chromatograms were run by the ascending technique[7]. Two sugars, arabinose and fructose, having similar R_F values were spotted at the origin of Eaton and Dikeman No. 613 papers, 23 cm in length, exposed 8 h to solvent vapors and irrigated at room temperature (26° ± 1°), with various water–ethanol–nitromethane solvents. After the solvents had reached the tops of the papers, the strips were withdrawn, air dried, and the sugar locations detected by developing the strips with the silver nitrate–sodium hydroxide reagents[8] by the dip technique. Another paper strip spotted with the two sugars was given two irrigations at room temperature with the solvent, water–ethanol–nitromethane (10:25:65 % by vol.), and developed as described above.

R_F, starting position and solvent proportions

To investigate the effect of starting position and solvent proportions on the R_F values for ascending chromatography, glucose and methionine were placed at various positions on Eaton and Dikeman No. 629 and 048 papers and irrigated with water–*tert.*-butanol and water–acetone solvents at 25° ± 0.1°, respectively. The same experiment was then repeated with varying solvent proportions which altered the single pass R_F values. Glucose was detected as described above and methionine was detected by spraying with ninhydrin[9].

RESULTS

Figs. 1 and 2 show reproductions of the developed chromatograms of arabinose and fructose after one and two ascents, respectively. The predicted difference in the centers of the spots after two ascents was calculated by eqn. (1) to be 1.8 cm and this is in moderate agreement with the observed separation of 2.0 cm.

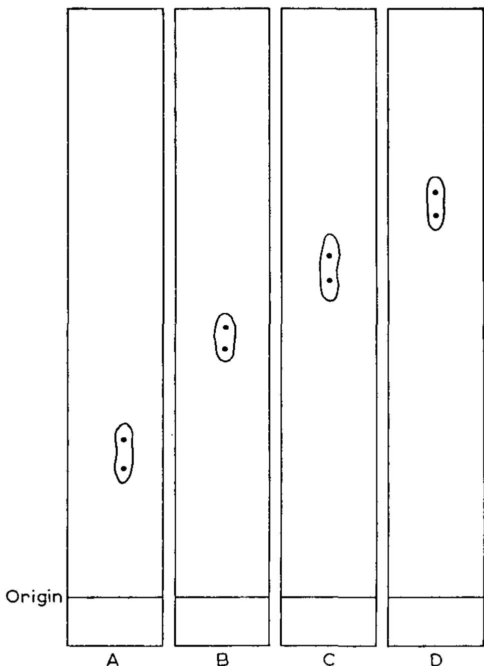

Fig. 1. Chromatography of arabinose and fructose with various water–ethanol–nitromethane solvents on Eaton and Dikeman No. 613 paper at 26° ± 1°. Arabinose is the more rapidly migrating component. The chromatograms were developed by the solvents listed (% by vol.).

Chromatogram	H_2O	*EtOH*	$MeNO_2$
A	10	25	65
B	12.1	39.4	48.5
C	17.6	42.8	39.6
D	24.1	47.0	28.9

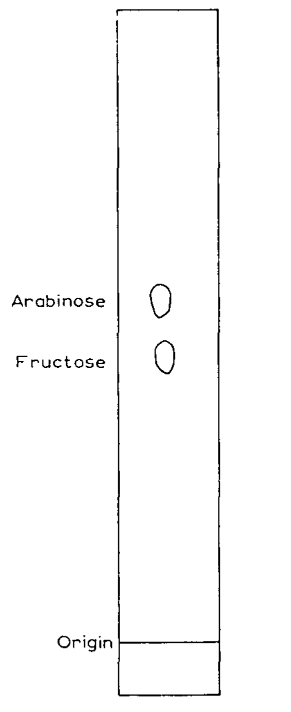

Fig. 2. Separation of arabinose and fructose with water–ethanol–nitromethane (10:25:65, % by vol.) by UMC with 2 solvent passes under conditions in Fig. 1.

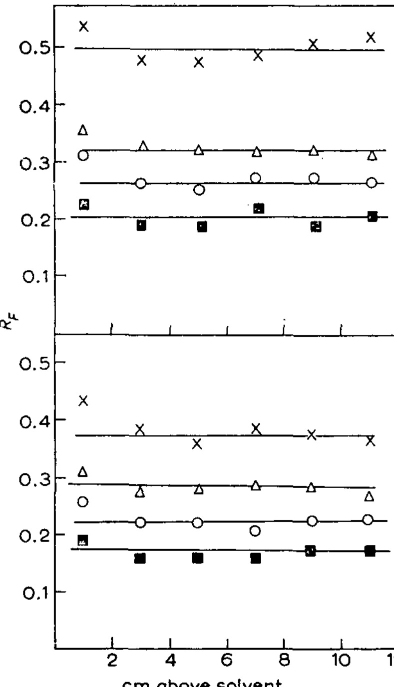

Fig. 3. Dependence of R_F on starting position on Eaton and Dikeman No. 629 paper. Upper plot, methionine; lower plot, glucose. Solvents are composed of water–*tert*.-butanol. Temperature was 25° ± 0.1°. Paper length was 23 cm (origin to top). % water by vol.: ■ 11.3; ○ 13.6; △ 16.1; × 23.1.

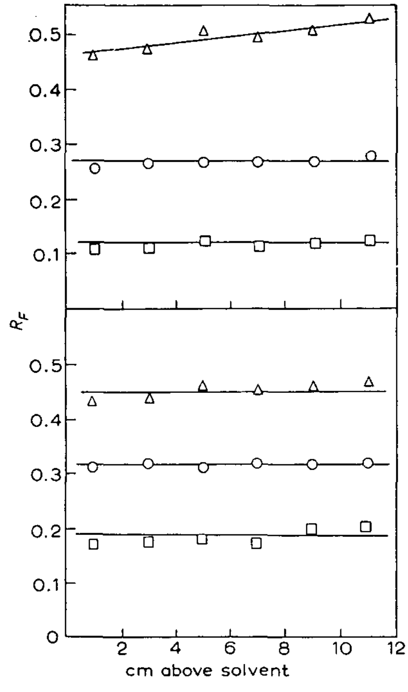

Fig. 4. Dependence of R_F on starting position on Eaton and Dikeman No. 048 paper. Conditions as in Fig. 3 except solvent was water–acetone. % water by vol.: □ 9.4; ○ 13.9; △ 16.5.

Fig. 3 depicts the dependence of the R_F value of glucose and methionine on their starting position on Eaton and Dikeman No. 629 paper with water–*tert.*-butanol as the irrigating solvent at 25° ± 0.1°. The ascending technique of chromatography was employed without prior exposure of the supports to solvent vapors.

Fig. 4 is a plot of the migration rates of glucose and methionine as a function of their starting position on Eaton and Dikeman No. 048 papers with water–acetone solvents at 25° ± 0.1°. The ascending technique of chromatography was used without prior exposure of the supports to solvent vapors.

The results of the calculations on the Indiana University IBM 650 computer relating the R_F values to the number of solvent passes required for a given degree of resolution ranging from 0.04 to 0.10 are listed in Tables I–IV. The degree of resolution, DR, is defined by eqn. (3):

$$DR = \frac{\Delta p}{L} \qquad (3)$$

where L is the length of the support in cm and Δp is the separation in cm of the two compounds after p passes.

DISCUSSION

Assumptions

In programming the IBM 650 computer to determine the minimum number of solvent irrigations required to separate two compounds by some preselected distance, it was assumed that R_F values were independent of paper position. A number of inconsistent reports about the effect of starting position on the R_F of various compounds have appeared in the literature[1–3,10,11]. Because of the wide variety of experimental conditions employed, inconsistent reports are not at all surprising. By and large, it has been our experience that when a solute is spotted on the lower portions of rectangular paper supports and irrigated with water miscible mixtures which do not produce R_F values in excess of 0.3–0.4 the R_F of the compound is relatively independent of starting position. With solvents producing R_F values below 0.2, practically no deviation in R_F was found with starting position. When papers of unusual geometries are used, particularly those which are fed with wicks or when the solutes are placed a long distance from the solvent, fluctuating R_F values are found[10,11]. R_F variation also seems to occur when irrigation with water immiscible systems is attempted[10].

In view of the observations that solvent gradients occur along the paper[10,12,13], particularly when using ascending chromatography, and because of the possible variations of the composition of solvents along the support[14], it was expected that the R_F value would appear to be relatively sensitive to starting position. It has been shown that the R_F function is influenced by at least three variables at constant temperature: the chemical potential of transport, the ratio of the cross-sectional areas of the stationary and mobile phases[15,16] and the ratio of the average velocity of the local solvent (velocity at solute band) to the velocity of the solvent front[10]. It seems entirely possible that as the height on the support increases, the ratio of the cross-sectional area decreases while the local solvent velocity increases because of solvent concentration gradients along the paper.The former effect would tend to decrease the migration rate of the band while the latter effect would tend to increase it. If these two variables of chromatography compensate each other quite closely, the net result

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TABLE I

NUMBER OF SOLVENT PASSES REQUIRED TO SEPARATE TWO SOLUTES 0.1 TIMES THE LENGTH OF THE SUPPORT

R_F of faster moving solute × 100	R_F of slower moving solute × 100								
	Number of solvent passes for required separation								
	2	*3*	*4*	*5*	*6*	*7*	*8*	*9–14*	*Impossible*
30	23–21								24–29
29	22–20								23–28
28	21–19	22							23–27
27	20–18	21							22–26
26	19–17	20							21–25
25	18–16	19							20–24
24	17–15	18							19–23
23	16–14	17	18						19–22
22	15–13	16	17						18–21
21	14–12	15	16						17–20
20	13–11	15–14							16–19
19	13–10	14							15–18
18	12–9	13			14				15–17
17	11–8	12		13					14–16
16	10–7	11	12						13–15
15	9–6	10	11						12–14
14	8–5	9	10						11–13
13	7–4	8	9			10			11–12
12	6–3	7	8		9				10–11
11	5–2	6	7	8					9–10
10	4–1	6–5		7					8–9
9	3–1	5–4		6					7–8
8	2–1	4–3		5				6	7
7	1	3–2	4				5		6
6		2–1	3			4			5
5		1	2			3			4
4			1		2			3	
3					1			2	
2								1	

To determine the number of passes for separation, locate R_F of slower moving compound in the row occupied by R_F of faster moving compound. The required number of passes is given by heading of column in which smaller R_F appears.

would lead to the observation of constant R_F values along the paper provided that the ratio of the transport potentials also remains constant.

Thus Tables I–IV will only give an exact value for p_{min} (minimum number of passes for preselected separation) when the R_F is constant; it will yield an approximate value when R_F is variable. It should be noted from eqn. (2) that no provision was made for "rounding off" the calculated difference between the two compounds after p irrigations. Consequently, unless the calculated separation is *exactly* equal to or greater than the preselected separation Δp, the computer will indicate separation is impossible regardless of how closely Δp and the calculated separation may agree. For example, if the extent of separation between two compounds was 0.079 the computer would indicate that a separation of 0.06 but not 0.08 could be achieved after an appropriate number of irrigations (computations made in increments of 0.02 for DR).

To demonstrate the relative constancy for R_F *versus* paper position for the type of systems described above (rectangular papers and water miscible solvents), glucose

TABLE II

NUMBER OF SOLVENT PASSES REQUIRED TO SEPARATE TWO SOLUTES 0.08 TIMES THE LENGTH OF THE SUPPORT

R_F of faster moving solute × 100	R_F of slower moving solute × 100 — Number of solvent passes for required separation: 2	3	4	5	6	7	8	9–14	Impossible
30	24–23								25–29
29	23–22	24							25–28
28	22–21	23							24–27
27	21–20	22							23–26
26	20–19	21							22–25
25	19–18	20							21–24
24	18–17	19							20–23
23	17–16	18							19–22
22	17–15		18						19–21
21	16–14		17						18–20
20	15–13	16							17–19
19	14–12	15							16–18
18	13–11	14							15–17
17	12–10	13							14–16
16	11–9	12		13					14–15
15	10–8	11		12					13–14
14	9–7	10	11						12–13
13	8–6	9	10						11–12
12	7–5	8	9						10–11
11	6–4	7	8						9–10
10	5–3	6	7				8		9
9	4–2	5	6			7			8
8	3–1	4	5		6				7
7	2–1	4–3			5				6
6	1	3–2		4					5
5		2–1		3				4	
4		1		2				3	
3				1				2	
2								1	

To determine the number of passes for separation, locate R_F of slower moving compound in the row occupied by R_F of faster moving compound. The required number of passes is given by heading of column in which smaller R_F appears.

and methionine were chromatographed as a function of starting position and solvent proportions. The data collected were graphed in Figs. 3 and 4. Methionine was selected as one of the solutes because in an earlier communication[3], it was noted that the behavior of this material on paper seemed to be anomalous when contrasted to that of other amino acids. Figs. 3 and 4 suggests that these peculiarities are not reflected as R_F variations with starting position.

On Eaton and Dikeman No. 629 paper there does appear to be a slight increase in the R_F values of the compounds when they are started very close to the solvent front (1 cm). Since the R_F which is determined on the first pass will be used to estimate the number of solvent passes required to separate the compounds, it is necessary that the measured R_F on a single pass be representative of that when compounds are started higher on the support. To fulfill this condition, the compound ought to be spotted 2–3 cm above the solvent.

Because the R_F value is somewhat influenced by loading, it is wise to use a

TABLE III

NUMBER OF SOLVENT PASSES REQUIRED TO SEPARATE TWO SOLUTES 0.06 TIMES THE LENGTH OF THE SUPPORT

R_F of faster moving solute × 100	R_F of slower moving solute × 100								
	Number of solvent passes for required separation								
	2	*3*	*4*	*5*	*6*	*7*	*8*	*9–14*	*Impossible*
30	25	26							27–29
29	24	25							26–28
28	23	24							25–27
27	23–22								24–26
26	22–21								23–25
25	21–20								22–24
24	20–19								21–23
23	19–18								20–22
22	18–17		19						20–21
21	17–16		18						19–20
20	16–15		17						18–19
19	15–14	16							17–18
18	14–13	15							16–17
17	13–12	14							15–16
16	12–11	13							14–15
15	11–10	12							14–13
14	10–9	11				12			13
13	9–8	10			11				12
12	8–7	9		10					11
11	7–6	8		9					10
10	6–5	7	8						9
9	5–4	6	7						8
8	4–3	5	6						7
7	3–2	4	5						6
6	2–1	3	4					5	
5	1	2	3					4	
4		1	2				3		
3			1			2			
2						1			

To determine the number of passes for separation, locate R_F of slower moving compound in the row occupied by R_F of faster moving compound. The required number of passes is given by heading of column in which smaller R_F appears.

solute concentration just above the limits of detection by the chromogenic reagents used for color development. The effects of loading on R_F are pictorially represented in a paper by STAHL AND KALTENBACH[17]. Reducing solute concentration will also help to minimize streaking.

Selection of p_{min}

Values of p_{min} were compiled for all combinations of R_F values ranging from 0.30 to 0.01. Larger values of R_F were not considered because the maximum extent of separation achieved by UMC occurs when the average apparent R_F of the two compounds is $1 - e^{-1}$ or 0.632 times the length of the support[3]. Thus, compounds having average R_F values in the range 0.3–0.4 will achieve maximum separation with two solvent passes and compounds having average R_F values greater than 0.4 will achieve maximum separation after one pass. Tables I–IV then will be most useful for the study of compounds possessing small R_F values; a condition necessary according to theoretical

TABLE IV

NUMBER OF SOLVENT PASSES REQUIRED TO SEPARATE TWO SOLUTES 0.04 TIMES THE LENGTH OF THE SUPPORT

R_F of faster moving solute × 100	R_F of slower moving solute × 100								
	Number of solvent passes for required separation								
	2	3	4	5	6	7	8	9–14	Impossible
30	27								28–29
29	26								27–28
28	25								26–27
27	24								25–26
26	23								24–25
25	22								23–24
24	21								22–23
23	20								21–22
22	19								20–21
21	18		19						20
20	17		18						19
19	16	17							18
18	15	16							17
17	14	15							16
16	13	14							15
15	12	13							14
14	11	12							13
13	10	11							12
12	9	10							11
11	8	9							10
10	7	8						9	
9	6	7				8			
8	5	6			7				
7	4	5			6				
6	3	4			5				
5	2	3		4					
4	1	2		3					
3		1		2					
2				1					

To determine the number of passes for separation, locate R_F of slower moving compound in the row occupied by R_F of faster moving compound. The required number of passes is given by heading of column in which smaller R_F appears.

considerations for the production of good resolution[3]. This phenomenon was predicted because a solvent which decreases the average R_F value of two compounds increases the ratio of the R_F values and is capable of discriminating more efficiently between them.

This experimentally supported prediction[3] made it possible to suggest a number of simple guiding rules for the selection of appropriate solvents to resolve closely related compounds. It was proposed for UMC that a solvent composition be chosen for reasons of economy, flow rate, etc. and the molar proportions altered to vary the R_F of the two components. The solvent should contain two miscible components; one in which the solutes are very soluble and the other in which the solutes are relatively insoluble. Appropriate proportions might then be selected which would achieve resolution of the compounds provided an appropriate number of irrigations were given. Tables I–IV help to quantitate these simple rules.

The techniques of UMC ought to prove valuable when it is known from chemical

experience that there exists in a mixture two or more closely related compounds which cannot be resolved with a single solvent irrigation or when a mixture is suspected from the appearance of an oblong spot on a chromatogram. To separate the mixture in the first instance, if pure materials are available, their R_F values can be determined and the number of solvent passes required to separate them found directly from Tables I–IV. If pure samples of the compounds are unavailable for chromatographic tests, then their resolution might be effected by following the guide lines above. The resolution of fructose and arabinose described below illustrates the utility of this suggested approach to UMC.

For the disengagement of these two compounds, a solvent system containing water–ethanol–nitromethane was selected because of its relatively rapid flow rate on paper. The molar proportions of water, ethanol and nitromethane were then altered so that the R_F values ranged from approximately 0.2 to 0.7. On these chromatograms, reproduced in Fig. 1, it will be noted that all the spots are oblong, indicative of a mixture, but complete separation is not achieved by any solvent mixture. It was assumed that the spots are composed of two bands whose centers are indicated by the dots. The R_F's of these dots were then measured and Tables I–IV were consulted for the appropriate number of solvent developments. It was learned that if a preselected degree of separation of 0.06 were required, the solvent, water–ethanol–nitromethane (10:25:65%, by vol.), giving chromatogram A ought to resolve the compounds after two solvent excursions. Fig. 2 shows that the compounds are well resolved as predicted and the extent of separation of the two spot centers is 2.0 cm which agrees reasonably well with that calculated from eqn. (1), 1.8 cm.

The selection of the extent of separation, Δp, which will be required to discern two visible spots is somewhat arbitrary, but a safe rule of thumb is that a 1.5–2 cm disengagement of spot centers will suffice. However, the practitioner will probably not experience much difficulty in the selection of Δp because previous experience with chromatography of related systems will probably furnish the necessary information. For the arabinose–fructose system described above, the extent of separation was arbitrarily set at 1.4 cm (experience indicated that 1.3–1.5 cm is generally sufficient for such systems) and since the paper is 23 cm long (origin to top), the relative separation (separation in cm/length of paper) is 1.4 cm/23 cm = 0.061. This value is approximately 0.06 and according to Table III for compounds having R_F values of 0.22 and 0.27, two solvent passes will be required. Other examples of the usefulness of UMC for the resolution of mixtures have been illustrated in a prior manuscript[3].

If the extent of separation required lies within the values tabulated, the required number of passes can be evaluated by interpolating. For example, if two compounds have R_F values of 0.07 and 0.05, for a separation of 0.09, it can be seen from Tables I and II that 7 passes will be required. Since the increments in Tables I–IV are small, interpolation ought to be accurate to within one pass. The number of solvent developments can be reduced if the bands are confined to small areas.

The degree of resolution, DR, required for separation will vary with experimental conditions and depend upon the length of the paper, extent of loading, sharpness of the zones, etc.[18–20]. The actual preselection of the required degree of resolution will be left to the judgment and previous experience of the investigator. A theoretical approach to the selection of DR is beyond the scope of this paper. For practical in-

stances, a theoretical evaluation of band dimensions seems impossible because the effects of adsorption, ion-exchange, etc. on band distortion cannot be predicted or anticipated. However, it should be pointed out, that on the second and subsequent passes during UMC, the solvent flows over the trailing edge of the band before reaching the leading edge. This effect aids in sharpening the bands. Therefore, when a solute moves an equal distance during continuous chromatography and UMC, the vertical spot dimensions on the latter chromatogram will be equal to or smaller than the vertical spot dimensions on the former chromatogram. This sharpening effect, of course, aids resolving the compounds.

Elongated spots which are often observed may be the result of adsorption, ion-exchange, non-linear isotherms, etc., which may cause a single pure material to appear as a distorted band. If the band is a single component, it will be expected that this distortion will become more extensive as the number of solvent passes is increased. On the other hand, if the solute is a mixture of more than one component, it ought to be possible to separate them with a sufficient number of solvent passes. Consequently, the probability of success in ascertaining the purity of the spot using UMC (provided the R_F values were small) would seem to be very high.

NOTE ADDED IN PROOF

After submission of this manuscript a related paper by LENK[21] came to my attention.

ACKNOWLEDGEMENTS

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SUMMARY

Tables relating the R_F value of two solutes to the number of solvent passes required for their separation by a preselected amount have been compiled for unidimensional multiple chromatography, UMC. These tables will help quantitate some simple guiding rules which were proposed for the selection of solvents for chromatographic separations. The use of the tables is illustrated and the rationale for the high probability of success of UMC is discussed.

REFERENCES

1 A. JEANES, C. S. WISE AND R. DIMLER, *Anal. Chem.*, 23 (1951) 415.
2 D. FRENCH AND G. WILD, *J. Am. Chem. Soc.*, 75 (1953) 2612.
3 J. A. THOMA, *Anal. Chem.*, 35 (1963) 241.
4 E. G. WOLLISH, S. MORTON AND H. HAWRYLYSHYN, *Anal. Chem.*, 33 (1961) 1138.
5 E. STAHL, *Angew. Chem.*, 73 (1961) 646.
6 F. H. HERNDON, H. E. APPERT, J. C. TOUCHSTONE AND C. N. DAVIS, *Anal. Chem.*, 34 (1962) 1061, and references therein.
7 S. ARONOFF, *Techniques of Radiobiochemistry*, Iowa State College Press, Ames, Iowa, 1956.
8 W. E. TREVYLAN, D. P. PROCTER AND J. S. MORRISON, *Nature*, 166 (1950) 444.
9 R. CONSDEN AND A. H. GORDON, *Nature*, 162 (1948) 180.

[10] J. C. GIDDINGS, G. H. STEWART AND A. L. RUOFF, *J. Chromatog.*, 3 (1960) 239, and references therein.
[11] N. KOWKABANY AND H. CASSIDY, *Anal. Chem.*, 34 (1952) 643.
[12] S. DIKSTEIN, *J. Chromatog.*, 2 (1959) 204.
[13] A. L. RUOFF, D. L. PRINCE, J. C. GIDDINGS AND G. H. STEWART, *Kolloid-Z.*, 166 (1959) 144.
[14] C. S. HANES, *Can. J. Biochem. Physiol.*, (and subsequent papers), 39 (1961) 119, and references therein.
[15] A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 35 (1941) 1358.
[16] R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
[17] E. STAHL AND V. KALTENBACH, *J. Chromatog.*, 5 (1961) 351.
[18] J. C. GIDDINGS AND R. A. KELLER, *J. Chromatog.*, 2 (1959) 626.
[19] J. C. GIDDINGS AND R. A. KELLER, *J. Chromatog.*, 2 (1959) 44.
[20] R. A. KELLER AND J. C. GIDDINGS, *J. Chromatog.*, 3 (1960) 205.
[21] H. P. LENK, *Z. Anal. Chem.*, 184 (1961) 107.

PAPER CHROMATOGRAPHY OF DYES

III. PAPER CHROMATOGRAPHY OF SOLUBILIZED VAT DYES

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INTRODUCTION

Chemically solubilized vat dyes, "Indigosols", are soluble derivatives of vat dyes. These solubilized dyes are sodium salts of sulphuric esters of the leuco form ($\geqslant$ C—OSO_3Na) of the vat dye. The reaction:

$$—CO— \longrightarrow —C(OH)= \longrightarrow —C(O—SO_3Na)=$$

for the preparation of these sulphuric ester salts of the leuco compounds may, in principle, be applied to all anthraquinoid and indigoid vat dyes.

Indigosols are very readily soluble in water. They are transformed to an insoluble form by oxidation in acid medium. During the process of oxidation the sulphuric esters of the leuco vat dyes are first hydrolysed, and then oxidized to the original parent dye. For example in the case of Indigosol Yellow V:

Up till now little information has been published on the chromatography of indigosols. RUGGLI AND STÄUBLE[1] chromatographed these dyes on a column of Al_2O_3 or $CaCO_3$. In our previous work[2,3] indigosols were chromatographed on paper by means of the ascending technique employing Whatman No. 1 paper and an aqueous pyridine solution as solvent. MATRKA, NAVRÁTIL AND FILIPI[4] employed paper chromatography to identify the dispersing and stabilizing agents present in indigosols (4-dimethylaminobenzenesulphonic acid, glucose, molasses, urea). Recently the paper chromatography of indigosols has been described by KOLŠEK, MLAKAR AND PERPAR[5]

who applied the ascending technique on SS 2043b paper with the following solvent systems: butan-1-ol–acetic acid–water (4:1:5) and butan-1-ol–pyridine–water (4:1:1).

In our institute paper chromatography of indigosols is performed by means of the descending technique, using as solvent system either methanolic ammonia solution, or a mixture of pyridine, isoamyl alcohol and ammonia, or a mixture of methanol and acetic acid. These solvent systems have also been used in centrifugal chromatography[6].

EXPERIMENTAL

(a) *Descending technique*

Whatman No. 1 paper was used, the size of the chromatograms being 160 × 470 mm with a running length of 400 mm. Spots of freshly prepared 1 % aqueous solutions of the dyes were applied on the start in 0.05 mg quantities by means of a micropipette. The chromatograms were developed at a temperature of 20–22°.

The following solvent systems were used:

S_1 = ammonia (25 %)–methanol–water (1:2:3),
S_2 = pyridine–isoamyl alcohol–ammonia (25 %) (1.3:1:1),
S_3 = methanol–acetic acid–water (4:1:1).

The R_F values with the S_1 system are dependent on the chemical structure of the dyes and on their functional groups and substituents. The S_2 system gives excellent separations suitable for estimating the purity and identity of the indigosols. The relation between the R_F value and the chemical structure of the dyes is, however, not as obvious as with the S_1 system. S_3 is suitable for some dyes only.

Acid solvent systems, although they find application for indigosols[5], are not suitable on account of the fact that during development of the chromatograms with acid systems a gradual hydrolysis of the sulphuric ester of the leuco dye takes place under formation of the monoester, which is in itself an inhibitor of oxidation.

The partially hydrolysed dye is exceedingly prone to autooxidation. In the course of the development of the chromatogram, autooxidation of the dye by atmospheric oxygen occurs under formation of semiquinone derivatives.

Alkaline systems preclude the possibility of autooxidation.

(b) *Centrifugal chromatography*

This was carried out with the pressureless apparatus with central spot development described previously by PAVLÍČEK, ROSMUS AND DEYL[7,8].

The chromatograms were cut from Whatman No. 3 paper and the separation was performed during 45 min at 600 r.p.m. The chromatograms were always developed with the same solvent system that was used for the descending technique.

With a few exceptions, the colour of indigosols is very faint, thus making detection of the chromatograms necessary. This can be performed by spraying the chromatogram with a warm (40°) solution of $NaNO_2$ (2 %) in 2 % hydrochloric acid. On spraying, immediate coloration of the dyes occurs, due to the oxidizing action of the reagent. It is also possible to utilize the high sensitivity of indigosols to autooxidation for detection purposes. The dried chromatograms are exposed to ultra-violet radiation and coloration of the dye will follow immediately.

TABLE I

CHROMATOGRAPHIC SEPARATION OF SOLUBILIZED VAT DYES

Colour index No.	*Commercial name**	*Solubilized form of the parent dye*	*R_F values and colour***		
			S_1	S_2	S_3
56006	Anthrasol Yellow HCG		0.70 Y 0.76 Y 0.82 Y 0.90 Y	0.43 Y 0.58 Y 0.65 GY 0.85 Y+	0.82 Y 0.86 GY — —
59051	Anthrasol Green I 3G		0.00 G 0.55 G 0.87 G+	0.00 G 0.60 G 0.65 G	0.00 G 0.55 G 0.62 G 0.72 G
59101	Anthrasol Yellow IGK		0.00 Y 0.16 Y 0.32 Y —	0.47 Y 0.53 Y 0.58 Y 0.80 Y+	0.52 Y — — —
59106	Tinosol Golden Yellow RK	Brominated CI 59101	0.00 Y 0.31 Y 0.87 Y+	0.57 Y 0.80 Y+ —	0.00 Y 0.26 Y 0.46 Y
59301	Cibantine Brilliant Orange RK		0.42 O	0.52 O	0.57 O
95316	Indigosol Red Violet IRRL		0.35 V	0.41 V	0.57 V
59706	Indigosol Golden Orange 12 R		0.26 O — — —	0.50 O 0.60 O 0.65 O —	0.00 O 0.07 O 0.32 O 0.40 O

(continued on p. 456)

TABLE I (*continued*)

Colour index No.	Commercial name*	Solubilized form of the parent dye	R_F values and colour**		
			S_1	S_2	S_3
59711	Indigosol Orange 18R		0.21 O — —	0.51 O 0.63 O —	0.00 O 0.26 O 0.35 O
59826	Cibantine Brilliant Green BF		0.00 G 0.03 G 0.10 G+ — —	0.00 G 0.37 G 0.53 G 0.65 G+ 0.75 G+	0.00 G 0.11 G 0.27 G — —
59831	Anthrasol Green IGG		0.00 G 0.02 G 0.05 G 0.38 B+ 0.50 B+ 0.60 B+	0.00 G 0.10 B 0.17 B 0.27 B+ 0.36 G+ 0.58 G	0.00 G 0.02 G 0.07 G 0.35 B — —
60011	Indigosol Brilliant Violet 14R		0.07 V — — — —	0.00 V 0.38 V+ 0.41 O+ 0.46 V+ 0.57 V	0.00 V 0.06 V 0.18 V 0.28 V —
60531	Tinosol Yellow V		0.00 Y 0.62 Y	0.55 Y 0.85 Y	0.00 Y 0.80 Y
61726	Indigosol Yellow 2GB		0.00 Y 0.85 Y — —	0.00 Y+ 0.56 Y 0.78 Y+ 0.86 Y+	0.00 Y 0.72 Y+ 0.85 Y —
69016	Soledon Brown R		0.00 Br 0.17 Br+ 0.62 Br+ 0.85 Br	0.00 Br+ 0.33 Br 0.40 Br+ 0.63 Br+	0.00 Br 0.73 Br — —

(*continued on p. 457*)

TABLE I (*continued*)

Colour index No.	Commercial name*	Solubilized form of the parent dye	R_F values and colour**		
			S_1	S_2	S_4
69501	Indigosol Olive Green IB		0.00 OG 0.30 OG — — —	0.00 Gr+ 0.02 OG+ 0.41 OG 0.46 OG 0.50 OG	0.00 OG 0.08 OG — — —
69826	Cibantin Blue CF		0.67 B — —	0.07 B 0.13 B 0.26 B	0.03 B 0.10 B 0.18 B+
70801	Cibantin Brown BR		0.00 Br 0.07 Br 0.16 Br 0.25 Br —	0.02 Br 0.06 Br 0.10 Br 0.20 Br 0.31 Br	0.00 Br 0.03 Br 0.51 Y+ —
73031	Indigosol Violet AZB		0.33 V —	0.37 Y+ 0.53 V	0.70 V —
73056	Indigosol OR		0.27 B 0.32 B+ 0.42 B 0.58 B	0.40 Y+ 0.47 B 0.52 B 0.58 B	0.61 B 0.71 B 0.78 Y+ —
73066	Indigosol O4B		0.27 B 0.55 V+	0.41 Y 0.58 B	0.72 B 0.78 Y+
73076	Indigosol O6B		0.22 B — —	0.43 B+ 0.60 B 0.78 Y+	0.65 B 0.71 B —

(*continued on p. 458*)

TABLE I (*continued*)

Colour index No.	Commercial name*	Solubilized form of the parent dye	R_F values and colour**		
			S_1	S_2	S_3
73336	Indigosol Orange HR		0.00 O+ 0.55 O — —	0.43 Y+ 0.52 O — —	0.00 O 0.46 O 0.58 O 0.75 O
73356	Indigosol Scarlet IB		0.45 S+ 0.56 S 0.65 S 0.87 Y+	0.00 S+ 0.37 Y+ 0.52 S 0.61 S	0.00 S 0.76 S — —
73361	Anthrasol Pink IR		0.00 P+ 0.70 P	0.00 P 0.61 P	0.00 P 0.72 P
73366	Indigosol Brilliant Pink 13B		0.00 P+ 0.70 P —	0.00 P 0.41 Y+ 0.62 P	0.00 P 0.82 P —
73386	Anthrasol Red Violet IRH		0.36 V 0.55 V — —	0.57 V — — —	0.27 V 0.38 V 0.66 V 0.75 V
73411	Anthrasol Brown IRRD		0.00 Br+ 0.48 Br —	0.00 Br+ 0.41 Br+ 0.56 Br	0.00 Br+ 0.71 Br —
73421	Indigosol HB		0.00 B+ 0.22 B — —	0.00 B+ 0.38 Y+ 0.46 V 0.56 B	0.00 B+ 0.57 B — —
73596	Anthrasol Printing Violet BBF		0.00 V+ 0.41 V —	0.41 Y+ 0.61 V —	0.00 V 0.70 V 0.80 Y+
73601	Anthrasol Printing Violet IRR		0.00 V+ 0.48 V 0.56 V+	0.58 V — —	0.00 V 0.75 V —

(*continued on p. 45*)

TABLE I (*continued*)

Colour index No.	Commercial name*	Solubilized form of the parent dye	R_F values and colour**		
			S_1	S_2	S_3
73671	Cibantine Grey BL		0.00 Gr 0.27 Gr 0.30 T	0.56 Gr — —	0.00 Gr 0.61 Gr 0.77 Y+
73801	Anthrasol Blue AGG		0.67 B	0.57 B	0.80 B
73821	Indigosol AZG		0.00 B+ 0.45 B —	0.43 Y+ 0.58 —	0.00 B 0.70 B 0.72 B
73831	Anthrasol Printing Black IB		0.00 Bl+ 0.28 Bl —	0.55 Bl — —	0.00 Bl 0.42 Bl 0.51 Bl

* Dye manufacturers names: Anthrasol: Farbwerke Hoechst A.G., Frankfurt/Main—Hoechst, Germany; Cibantine: Ciba Ltd., Basle, Switzerland; Indigosol: Durand & Huguenin S.A., Basle, Switzerland; Soledon: Imperial Chemical Industries Ltd., Dyestuffs Division, Manchester, England; Tinosol: J. R. Geigy S.A., Basle, Switzerland.

** Solvents: S_1 = 25% ammonia–methanol–water (1:2:3); S_2 = Pyridine–isoamyl alcohol–25% ammonia (1.3:1:1); S_3 = Methanol–acetic acid–water (4:1:1).

Abbreviations: B = blue; Bl = black; Br = brown; G = green; Gr = grey; O = orange; OG = olive; P = pink; S = scarlet; T = turquoise; V = violet; Y = yellow; + = spot hardly visible

RESULTS AND DISCUSSION

Some 160 commercial brands of indigosols were analysed by means of the descending chromatographic technique described above. Chromatographically interesting dyes were also chromatographed centrifugally in order to compare the separations achieved with the two techniques. The results of the descending chromatographic technique are shown in Table I. This table contains most of the important dyes of known constitution.

Figs. 1, 2 and 3 illustrate the chromatographic separation of artificially prepared mixtures of indigosols.

A comparison of the descending and centrifugal chromatograms shows that there is good agreement in the separating power of the two techniques. The sequence of the dyes is identical in the two cases. The chromatograms were developed with the solvent system S_1.

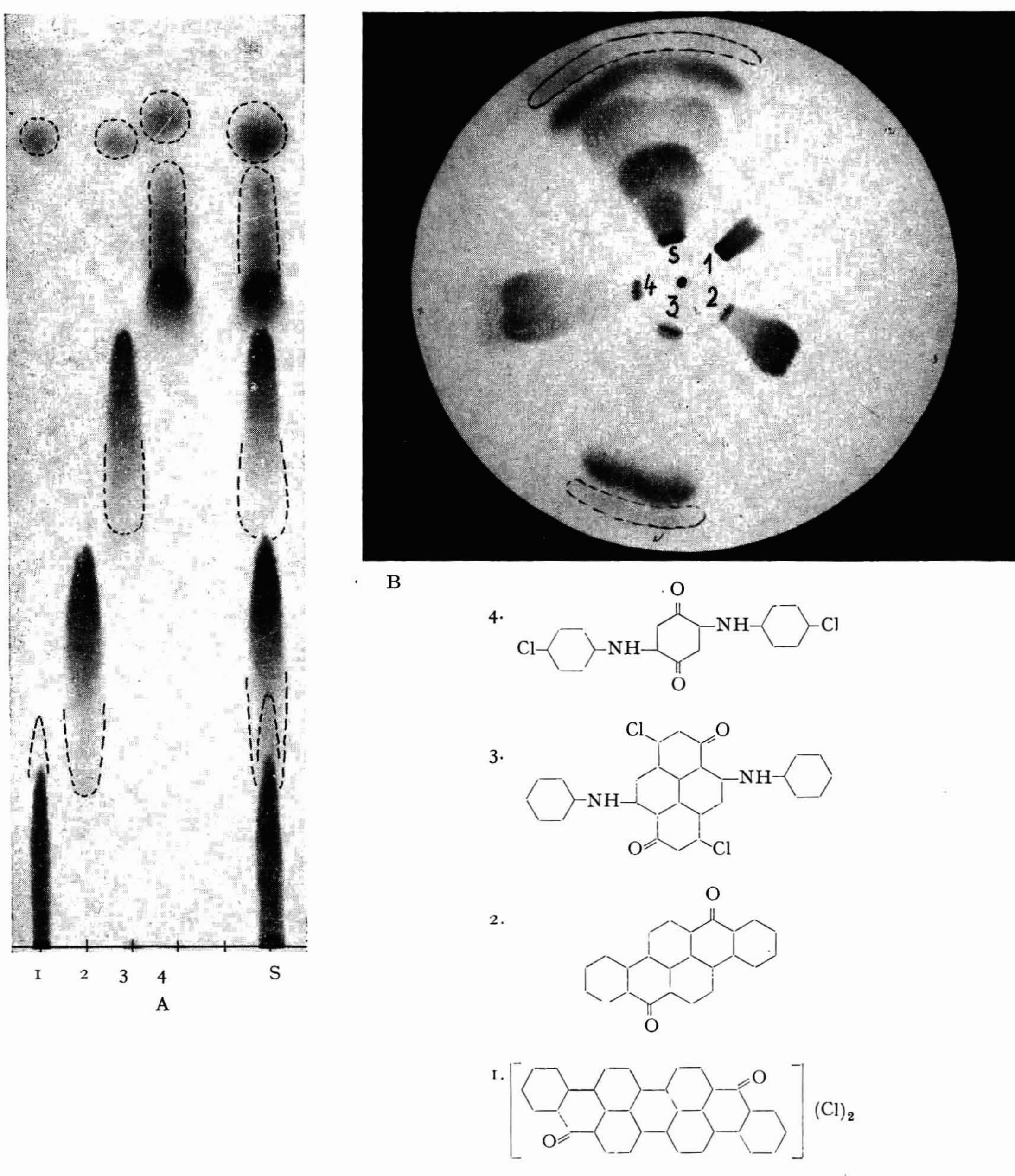

Fig. 1. Example of a chromatographic separation of a mixture of solubilized vat dyes. A. Descending technique. B. Centrifugal technique. 1 = Indigosol Brilliant Violet 14R; 2 = Anthrasol Yellow IGK; 3 = Anthrasol Green 13G; 4 = Anthrasol Yellow HCG; S = mixture of 1,2,3 and 4.

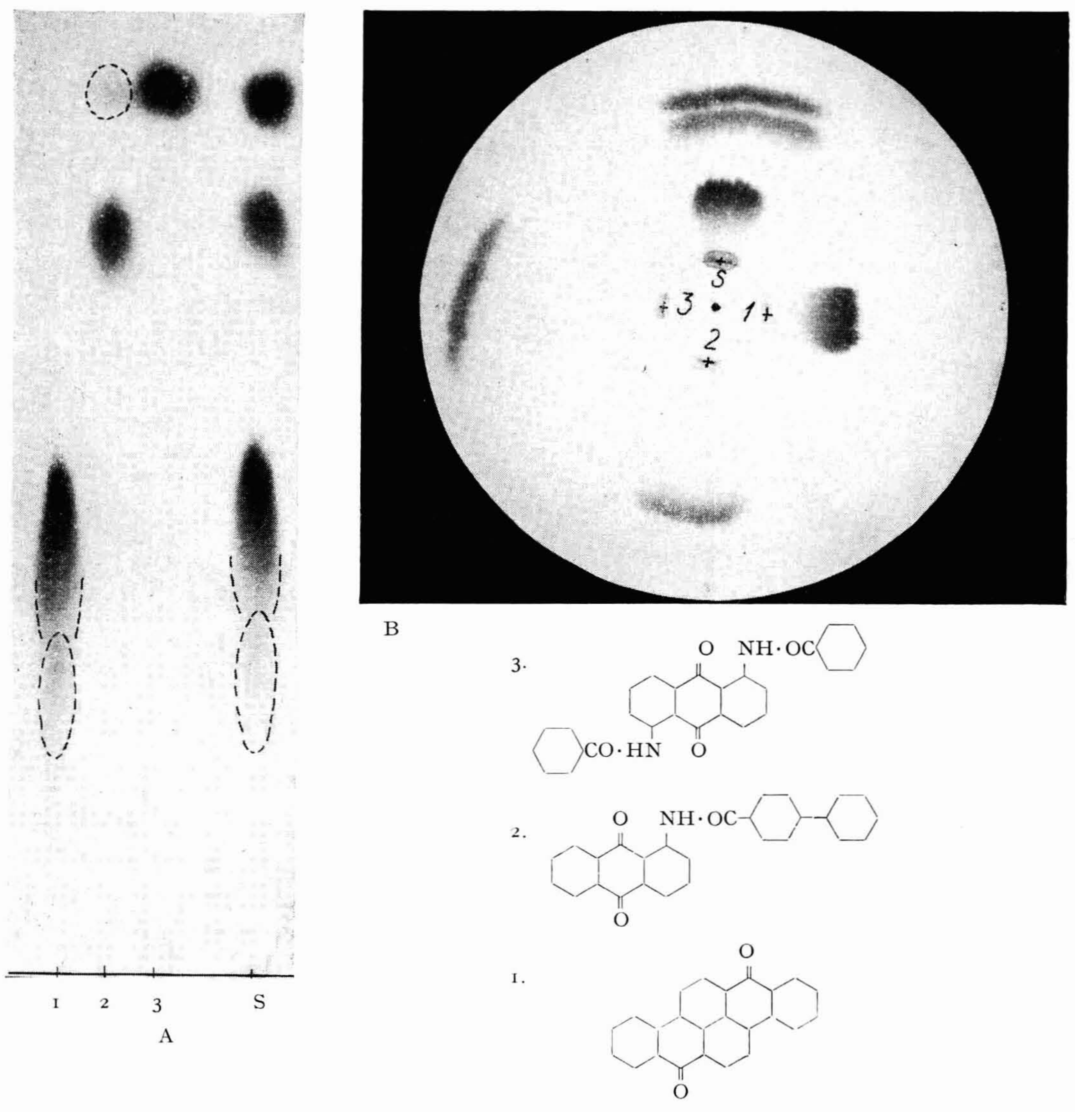

Fig. 2. Example of a chromatographic separation of a mixture of solubilized vat dyes. A. Descending technique. B. Centrifugal technique. 1 = Indigosol Golden Yellow IGK; 2 = Tinosol Yellow V; 3 = Indigosol Yellow 2GB; S = mixture of 1, 2 and 3.

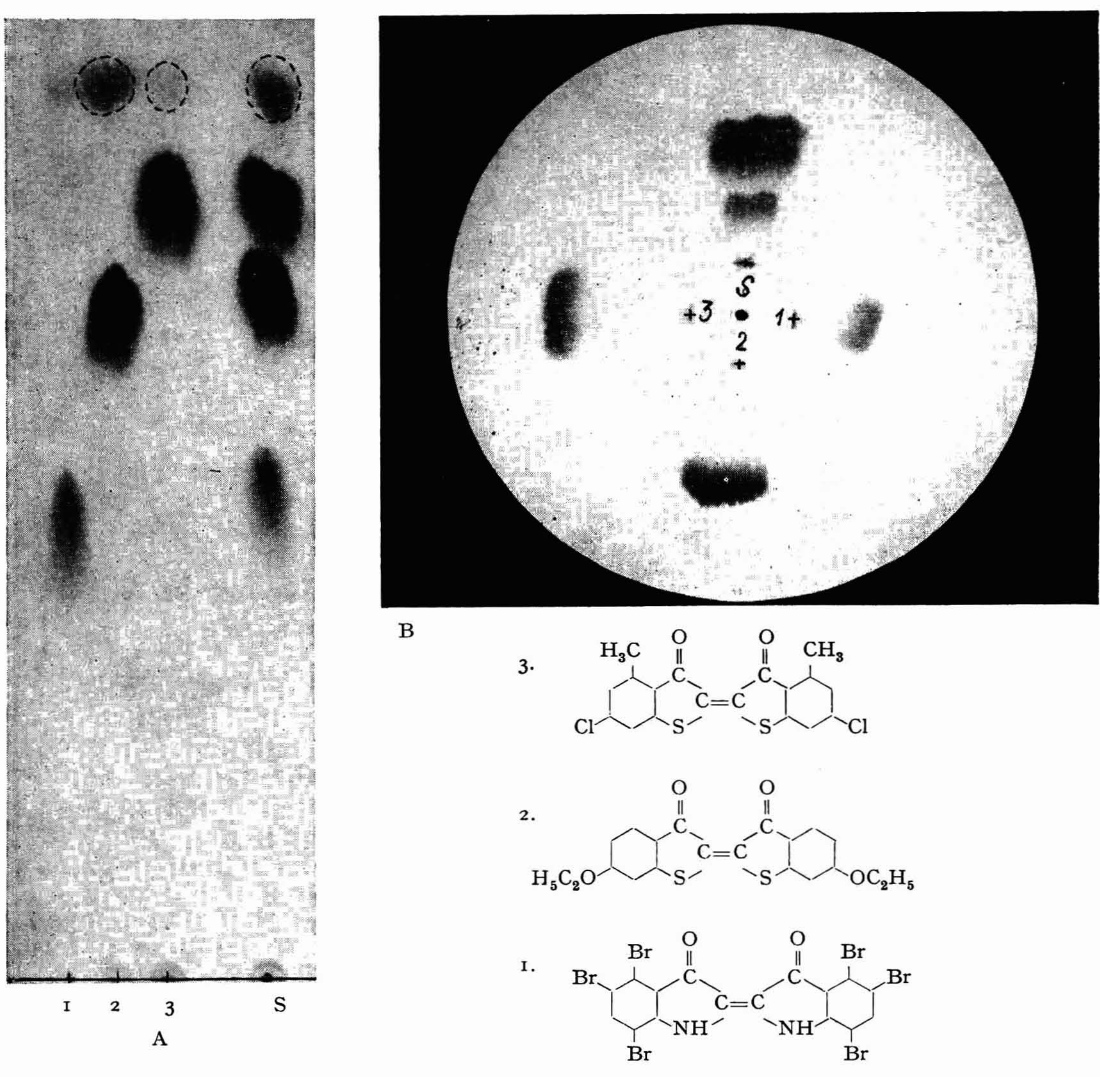

Fig. 3. Example of a chromatographic separation of a mixture of solubilized vat dyes. A. Descending technique. B. Centrifugal technique. 1 = Indigosol O6B; 2 = Indigosol Orange HR; 3 = Anthrasol Pink IR; S = mixture of 1, 2 and 3.

With solvent system S_1, the following relationships were established between the chromatographic behaviour of the solubilized vat dyes and their chemical structure:

(1) In all instances it is obvious that the chromatographic behaviour of the vat dyes (parent dyes) is analogous, the size of the dye molecule being of decisive influence. Dyes with a simple structure possess the highest R_F value, and this decreases with increasing size of the molecule as a result of increasing dye adsorption.

(2) Simpler indigoid, thioindigoid and arylaminoquinone dyes have a higher R_F than the more complex anthraquinone dyes.

(3) Thioindigoid dyes have a higher R_F than indolethionaphthene dyes and miscellaneous dyes.

(4) Symmetrical indigoid dyes usually have a higher R_F than asymmetrical dyes.

(5) Dibenzanthrones have a higher R_F value than isodibenzanthrones.

(6) In the case of dyes with the same carbon skeleton, an increase in the number of substituents, *e.g.* —Cl, —Br, —CH_3, —OCH_3, causes the R_F value to decrease.

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SUMMARY

Solubilized vat dyes were separated by paper chromatography, using a methanolic solution of ammonia as solvent system. A system containing pyridine, isoamyl alcohol and ammonia, as well as a mixture of methanol and acetic acid were also used. Both descending and centrifugal chromatography were employed. The relation between dye constitution and chromatographic behaviour has been examined.

REFERENCES

1 P. RUGGLI AND M. STÄUBLE, *Helv. Chim. Acta*, 23 (1940) 689.
2 J. ŠRÁMEK, *Textil*, 13 (1958) 346.
3 J. ŠRÁMEK, *Paper presented at the 1st Conference V T S* (Czechoslov. Scientific Technical Organization), Brno, Nov. 1958.
4 M. MATRKA, F. NAVRÁTIL AND J. FILIPI, *Chem. Prumysl*, 7 (1957) 343.
5 J. KOLŠEK, F. MLAKAR AND M. PERPAŘ, *Z. Anal. Chem.*, 188 (1962) 345.
6 J. ŠRÁMEK, *Textil*, 17 (1962) 147.
J. ŠRÁMEK, *Paper presented at the Symposium on Chromatography in the Centrifugal Field*, Prague, Feb. 1962.
7 M. PAVLÍČEK, J. ROSMUS AND Z. DEYL, *J. Chromatog.*, 7 (1962) 19.
8 M. PAVLÍČEK, J. ROSMUS AND Z. DEYL, *J. Chromatog.*, 9 (1962) 92.

DIE TRENNUNG NEUTRALER UND BASISCHER COBALAMINE AN IONENAUSTAUSCHERPAPIEREN

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Für die Trennung von wasserlöslichen Vitaminen sind mit gutem Erfolg Ionenaustauscherpapiere eingesetzt worden[1]. Analog zu den an Säulen gefundenen Bedingungen[2] waren auch für die Papierchromatographie schwach saure Ionenaustauscherharze am besten geeignet. Anschliessend verwerteten wir unsere Ergebnisse zur Analyse kompletter Vitamin-B_{12}-Arten. Die Ladungsunterschiede zwischen neutralen und basischen Cobalaminen liessen dabei ausgeprägte Trenneffekte erwarten.

Neben Cyanocobalamin hat in der letzten Zeit das positiv geladene Aquocobalamin (= Vitamin B_{12b}) medizinische und pharmazeutische Bedeutung erlangt[3,4]. Eine rasche und sichere Identifizierung und Differenzierung beider Cobalamine sollte erreicht werden, die auch den Nachweis gegenseitiger Verunreinigung ermöglichte. Wir verwendeten dafür Amberlite-Ionenaustauscherpapiere*.

METHODIK

Die Papiere wurden vor ihrem Einsatz besonders präpariert, die Austauscherharze wurden mit bestimmten Gegenionen beladen bzw. auf bestimmte pH-Werte eingestellt. Bei Kationenaustauscherpapieren (SA und WA) erfolgte die Behandlung mit *N* Salzsäure oder *N* Natriumchloridlösung, bei Anionenaustauscherpapieren (SB und WB) mit *N* Natronlauge bzw. *M* Lösungen verschiedener Säuren. Als Puffer dienten Acetat-Pufferlösungen von pH 3.5–6.0.

Zum Beladen bzw. Puffern wurden die Papierstreifen 60 Min. in die Lösungen eingelegt; bei längerer Behandlungszeit beobachteten wir eine Beeinträchtigung der Cobalamintrennung und sogenannte Schwanzbildung. Die feuchten Papiere wurden über Nacht (an der Luft bei Zimmertemperatur) getrocknet und am nächsten Tag verwendet. Am Startpunkt wurden 1–3 μg Cyanocobalamin und/oder Aquocobalamin aufgetragen (Lösungen mit 50 μg/ml). Die Chromatographie erfolgte absteigend, in abgedeckten Glasgefässen unter Lichtschutz. Als Laufmittel verwendeten wir Wasser oder ein Gemisch aus Dioxan–*N* Salzsäure–Wasser. Die Laufstrecke der Lösungsmittelfront betrug 20–25 cm.

Eine Entwicklung der Papiere konnte durch die Eigenfärbung der Verbindungen gut verfolgt werden. Nach dem Trocknen an der Luft eignete sich auch die Auswertung im U.V.-Licht. Dabei konnten selbst die im sichtbaren Licht nicht mehr wahrnehmbaren Flecke noch gut erkannt werden. Häufig wurden ausserdem, besonders beim

* Dem Serva-Entwicklungslabor, Heidelberg danken wir für die freundliche Überlassung einiger Muster.

Vorliegen von Aquocobalamin, am Startpunkt stark fluoreszierende Flecke festgestellt. Die Natur und Herkunft dieser Begleitsubstanzen blieb uns unbekannt. Ein anderer bemerkenswerter Befund bestand darin, dass Cyanocobalamin bei der Chromatographie häufig in zwei parallellaufende Flecke aufspaltete. Auch für dieses Ergebnis haben wir noch keine befriedigende Erklärung gefunden. Bekanntlich wird diese Erscheinung in der allgemeinen Papierchromatographie nicht beobachtet.

Die quantitative Auswertung der Trennungen haben wir wie folgt vorgenommen: Die Papiere wurden auf pH 4.6 oder 6.0 gepuffert. Von einer wässrigen Lösung wurde ein genau gemessenes Volumen, das etwa je 100 μg Cyano- und Aquocobalamin enthielt, aufgetragen. Die Chromatographie erfolgte mit Wasser oder Dioxan–Salzsäure–Wasser als Laufmittel. Nach der Trennung wurden die Flecke ausgeschnitten und dreimal mit einer Lösung von 7.5 g Natriumchlorid in 100 ml Aceton–Wasser-Gemisch 1:1 eluiert; Aquocobalamin wurde zuvor durch Besprühen mit einer *M* Kaliumcyanidlösung in Cyanocobalamin übergeführt. Die vereinigten Eluate versetzten wir mit 1 ml *N* Essigsäure, um den für die Existenz des neutralen Cobalamins optimalen pH-Wert einzustellen, und ergänzten das Gesamtvolumen auf 10 ml.

Die Gehaltsbestimmung erfolgte spektrophotometrisch bei 361 mμ gegen das entsprechende Elutionsmittel. Für die Messungen wurde ein Universal-Spektrophotometer VSU 1 des VEB Carl Zeiss Jena verwendet.

ERGEBNISSE

Nach der angegebenen Methode wurden die Trenneffekte an verschiedenen Ionenaustauscherpapieren ermittelt. Die Ergebnisse sind in Tabelle I, II und III dargestellt.

DISKUSSION

Unsere Ergebnisse stimmen in den wesentlichen Punkten mit den an Ionenaustauscher-*säulen* gewonnenen Aussagen[5–8] überein.

Die eingesetzten Cobalamine werden durch Anionenaustauscherharze erwartungsgemäss nicht gebunden (Tabelle I). An stark sauren Kationenaustauscherpapieren treten andererseits Elutionsschwierigkeiten auf. Für die angestrebte Trennung eignen sich nur schwach saure Austauscherharze (Tabelle II, Fig. 2). Während aber bei der Säulenchromatographie die Harze vorwiegend in der H^+-Form eingesetzt werden, erweist sich bei der Papierchromatographie eine Pufferung erforderlich. Die Trennwirkung des Papiers steigt mit dem pH-Wert des Puffers, wenn die bewegliche Phase lediglich aus Wasser besteht. Sie erreicht am Neutralpunkt ihr Optimum. Eine graphische Darstellung der Abhängigkeit zwischen R_F- und pH-Wert demonstriert (Fig. 1), dass sich die Kurve an den Grenzen des Stabilitätsgebietes von Cyanocobalamin[9] dem R_F-Wert 0 bzw. 1 nähert. Sie erreicht den Wert 0 beim Übergang des neutralen in ein basisches Cobalamin, und sie steigt auf den Wert 1 bei der Ausbildung eines sauren Cobalamins.

In der Chromatographie an Ionenaustauscherpapieren kann reines Wasser als Laufmittel Verwendung finden. Diese Vereinfachung ermöglicht eine wesentlich günstigere Arbeitstechnik als sie an normalen Papieren mit zusammengesetzten organischen Lösungsmitteln üblich ist. Bei Benutzung von Laufmitteln aus mehreren Komponenten erhält man sehr unterschiedliche Ergebnisse. Erfolgt die Trennung mit

TABELLE I

TRENNUNGEN AN ANIONENAUSTAUSCHERPAPIEREN

Papier: Amberlite SB-2 (strong basic, Typ Amberlite IRA-400) bzw. Amberlite WB-2 (weak basic, Typ Amberlite IR-45).Laufmittel: Wasser.

Beladungsform	R_F	
	SB-2	*WB-2*
	CN-B_{12} und H_2O-B_{12}	*CN-B_{12} und H_2O-B_{12}*
OH^-	0.96	0.80
CH_3COO^-	0.96	0.94
BO_3^{3-}	0.92	0.83
Cl^-	0.96	0.92

TABELLE II

TRENNUNGEN AN KATIONENAUSTAUSCHERPAPIEREN

Papier: Amberlite WA-2 (weak acid, Typ Amberlite IRC-50). Laufmittel: Dioxan–*N* Salzsäure–Wasser.

Mischungsverhältnis Dioxan–Salzsäure–Wasser	R_F			
	pH 4.6		*pH 6.0*	
	CN-B_{12}	*H_2O-B_{12}*	*CN-B_{12}*	*H_2O-P_{12}*
30: 5:65	0.68	0.27	0.91	0
30:10:60	0.45	0.18	0.90	0.04
60:10:30	0.78	0.39	0.93	0.20
80:10:10	0.66	0.59	0.96	0.82

TABELLE III

QUANTITATIVE TRENNUNGEN

Papier: Amberlite WA-2.
Laufmittel: Wasser oder Dioxan–*N* Salzsäure–Wasser.

Pufferung pH	*Laufmittel*	*µg eingesetzt*		*% gefunden*	
		CN-B_{12}	*H_2O-B_{12}*	*CN-B_{12}*	*H_2O-B_{12}*
6.0	Wasser	100	100*	79.0	96.5
6.0	Wasser	156	123	83.1	79.5
6.0	Dioxan–Salzsäure–Wasser (3:1:6)	100	123	81.8	74.8
4.6	Wasser	156	123	86.2	70.4

* Aquo-cytobion.

einem Aceton–Wasser-Gemisch, so wird die Wanderungsgeschwindigkeit des Cyanocobalamins im Vergleich zu reinem Wasser auf ein Drittel herabgesetzt und Aquocobalamin verbleibt am Startpunkt. Der Zusatz von Neutralsalzen, Natriumacetat oder -cyanid, hat auf die Trennung keinen signifikanten Einfluss. In dem System Dioxan–Salzsäure–Wasser wandert jedoch auch das Aquocobalamin. Dabei kommt es

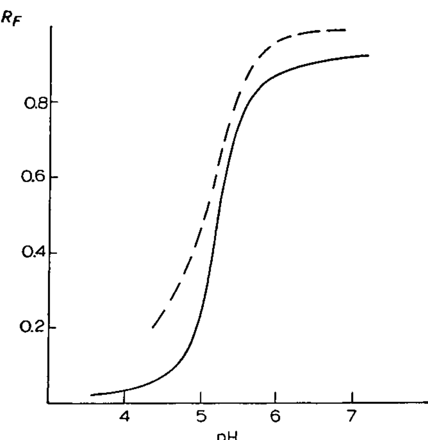

Fig. 1. Der Einfluss der Pufferung auf den R_F-Wert des Cyanocobalamins und Cobinamids bei der Chromatographie an Amberlite WA-2. ——— Cyanocobalamin (Wasser); — — — — Cobinamid (Dioxan–N Salzsäure–Wasser, 3:1:6).

zu einer Auftrennung des Lösungsmittelgemisches während der Wanderung. Das Cyanocobalamin bewegt sich in der schwach sauren Komponente (pH 4.3), das Aquocobalamin dagegen an der scharf abgegrenzten Front einer sauren Phase (pH 2.0) (Fig. 2). Bei optimaler Pufferung des Papiers auf pH 6.0 stimmen daher die mit Wasser und die mit Dioxan–Salzsäure–Wasser erhaltenen R_F-Werte des Cyanocobalamins

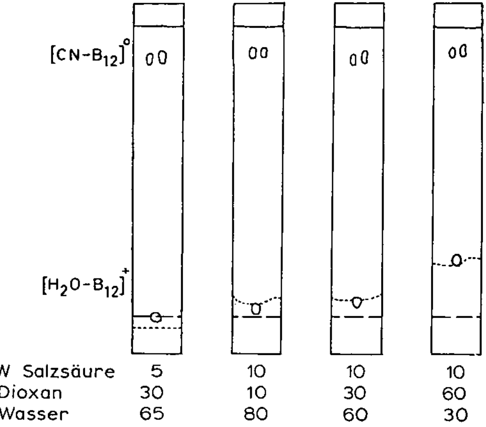

Fig. 2. Die Trennung von Aquo- und Cyanocobalamin an Amberlite WA-2 (Pufferung pH 6.0) unter Verwendung verschiedener Salzsäure–Dioxan–Wasser-Gemische als Laufmittel. — — — Startlinie; ——— Front der Lösungsmittelkomponente pH 4.3; - - - - Front der Lösungsmittelkomponente pH 2.0.

überein. Den schärfsten Trenneffekt erzielt man mit Papier vom pH 4.6 unter Verwendung eines Dioxan–Salzsäure–Wasser-Gemisches (3:1:6).

Grundsätzlich gleiches Verhalten wie Cyanocobalamin zeigt die inkomplette Vitamin-B_{12}-Art Cobinamid (= Ätiocobalamin, Faktor B, Faktor I). An einem schwach sauren Ionenaustauscherpapier liegen die R_F-Werte mit einem Dioxan–Salzsäure–Wasser-Gemisch höher als mit Wasser, sie steigen korrespondierend mit dem pH-Wert (Fig. 1).

Aus den Angaben über die quantitative Erfassung der getrennten Cobalamine geht hervor (Tabelle III), dass die Bestimmung noch unbefriedigende Ergebnisse liefert. Wie die allgemeine Papierchromatographie wird auch die Chromatographie an Ionenaustauscherpapieren in erster Linie als eine qualitative Methode einzuschätzen sein.

ZUSAMMENFASSUNG

Die Trennung von Cyano- und Aquo- bzw. Hydroxocobalamin wurde an verschiedenen Ionenaustauscherpapieren und unter verschiedenen Bedingungen untersucht. Die Ergebnisse einer qualitativen und quantitativen Methode werden mitgeteilt und diskutiert. Ähnliche Angaben liegen über das Verhalten von Cobinamid vor.

SUMMARY

The separation of cyano- and aquocobalamin on different ion-exchange papers using different conditions was investigated. The results of a qualitative and a quantitative method are described and discussed. Similar data are given for the behaviour of cobinamide.

LITERATUR

1 R. HÜTTENRAUCH UND L. KLOTZ, *Experientia*, 19 (1963) 95.
2 W. POETHKE UND L. KLOTZ, *Pharm. Zentralhalle*, in Vorbereitung.
3 H. C. HEINRICH, Vitamin B_{12} und Intrinsic Faktor, 2. *Europäisches Symposion, Hamburg 1961, Stuttgart* 1962.
4 E. L. SMITH, J. L. MARTIN, R. J. GREGORY UND W. H. C. SHAW, *Analyst*, 87 (1962) 183.
5 M. M. MARSH UND N. R. KUZEL, *Anal. Chem.*, 23 (1951) 1773.
6 K. H. MENKE, *Naturwissenschaften*, 45 (1958) 263.
7 P. J. VAN MELLE, *J. Am. Pharm. Assoc., Sci. Ed.*, 45 (1956) 26.
8 M. M. SAKOTA, M. IWAKE, M. YAMAMOTO UND H. KOSAKA, *Vitamins (Kyoto)*, 9 (1955) 302; *C.A.* 50 (1956) 16033.
9 R. HÜTTENRAUCH UND A. DÖLL, *Pharm. Zentralhalle*, 101 (1962) 549.

ANALYSE VON POLYPHENYLGEMISCHEN MIT DER DÜNNSCHICHTCHROMATOGRAPHIE

I. QUALITATIVE ARBEITEN

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A. EINLEITUNG

Das rasch steigende Interesse an der Verwendung von organischen Gemischen auf Polyphenylbasis* zur Reaktorkühlung und/oder -moderierung, verlangt die Ausarbeitung geeigneter Analysenmethoden. Durch Radio- und Pyrolyse bei Temperaturen um 400° entstehen während des Reaktorbetriebes aus dem verhältnismässig einfachen Gemisch der drei Terphenyle und des Biphenyls äusserst komplexe Vielstoffgemische**, deren Zusammensetzung zu kennen, sowohl für den Verfahrensingenieur als auch für den Chemiker, z.B. zur Aufstellung von Reaktionsmechanismen von grosser Bedeutung ist.

Unter den von den verschiedenen Forschungsgruppen angewandten Analysenmethoden hat sich die Hochtemperaturgaschromatographie als die vielseitigste und erfolgreichste erwiesen. Nichtdestoweniger haben eine Reihe anderer Methoden wie U.V., I.R., Röntgenstreuung[1] und Massenspektrometrie[2,3] ihren Platz.

Über die Anwendung anderer chromatographischer Trennmethoden finden sich in der Literatur nur spärliche Angaben: Eine spanische Arbeitsgruppe[4] versuchte nach Vorschriften von SPOTSWOOD[5] Trennungen der Terphenyle und des Biphenyls auf teilazetyliertem Papier mit sehr schlechtem Trennresultat. Die Reihenfolge der Elution ist merkwürdig (nach zunehmendem R_F-Wert): *p*-Terphenyl, Biphenyl, *m*-Terphenyl, *o*-Terphenyl.

WEST[6] UND HELLMAN und Mitarb.[7] studierten eine säulenchromatographische Trennung von niederen Polyphenylen. Auch diese Ergebnisse waren wenig überzeugend. Eigene Versuche mit der Säule waren im Wesentlichen nur für die Reinigung ausgewählter Polyphenyle erfolgreich.

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* Gruppe kettenförmiger organischer Verbindungen mit der Struktur C_6H_5–$(C_6H_4)_n$–C_6H_5 mit $n \geqslant 0$, nebst Verzweigungen.

** Wasserstoff, die niederen aliphatischen Kohlenwasserstoffe bis etwa Butan, Benzol, Toluol, *m*- und *p*-Xylol, alkylsubstituierte Biphenyle und Terphenyle, Quater-, Quinqua- und Sexiphenyle, sowie längere Ketten mit Häufigkeitsmaxima bei Dreiersequenzen (wenn Terphenyle eingesetzt werden), Phenanthren, Triphenylen und andere noch nicht identifizierte kondensierte Aromaten, sowie teilhydrierte und vor allem alkylsubstituierte Produkte der genannten kondensierten Aromaten.

B. SICHTBARMACHUNG UND IDENTIFIZIERUNG

1. Dünnschichtchromatographie

Auf diesen Erfahrungen fussend, bot sich die Dünnschichtchromatographie (DSC) an, bei der die Trägerschicht gegen aktive Sprühreagenzien beliebig beständig ist. Die besten Trennungen wurden auf „Aluminiumoxid G, Merck" (nach STAHL) erhalten (auf Einzelheiten wird später eingegangen).

2. Nachweis der Reinstoffe

In Tabelle I sind die Reinstoffe zusammengestellt, die in dieser Arbeit untersucht werden. Tabelle II zeigt eine Übersicht über die Ergebnisse der Versuche zur Sicht-

TABELLE I

ÜBERSICHT UND FORMELBILDER DER UNTERSUCHTEN POLYPHENYLE

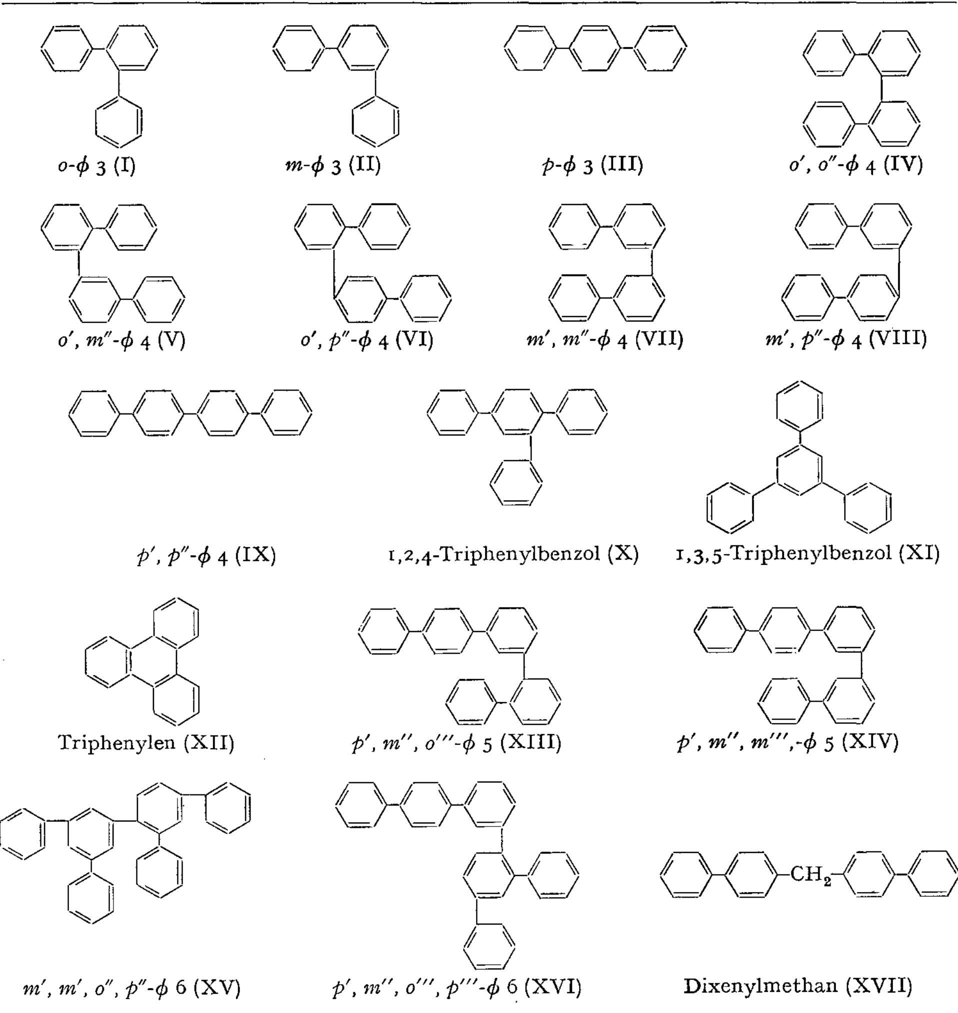

TABELLE II

SICHTBARMACHUNG VON POLYPHENYLEN UND ERFASSUNGSGRENZE DER INDIKATOREN

	Unbesprüht				*Besprüht mit*													
	U.V.-Licht				H_2SO_4-HCHO[8]		$SbCl_5$		HNO_3		$Ce(SO_4)_2$–HNO_3 konz.		$Ce(SO_4)_2$–HNO_3 konz. U.V.-Licht 366 mμ		Tetracyanäthylen[10,24]		Tetrachlorphthalsäureanhydrid[5]	
	254 mμ		*366 mμ*															
	*Farbe**	*Empf.**	*Farbe*	*Empf.*	*Farbe*	*Empf.*	*Farbe*	*Empf.*	*Farbe*	*Empf.*	*Farbe*	*Empf.*	*Farbe*	*Empf.*	*Farbe*	*Empf.*	*Farbe*	*Empf.*
o-φ3	d	1	grau	1	grau	10	grün	1	d	10	d	10	grau	0.01	rot	10	h	1
m-φ3	h	0.1	d	1	rot	1	rot	1	rot	1	rot	0.1	rot	0.01	rot	10	h	1
p-φ3	h	0.1	d	1	grau	1	braun	1	d	1	d	1	gelb	0.01	viol.	10	h	1
Triphenylen	h	0.1	d	1	blau	1	grün	1	grau	1	d	0.1	hell-blau	0.01	—	—	h	1
p′,m″,o‴,p‴′-φ6	h	0.1	d	1	braun	1	gelb	1	grün	1	d	0.1	d	0.01	—	—	—	—

* d = Fleck dunkler als Untergrund; h = Fleck heller als Untergrund. Empfindlichkeit in γ.

barmachung auf der DSC-Platte. Die beschriebenen Farben verändern sich etwas mit der aufgetragenen Menge und dem Schichtmaterial. Der Nachweis von Biphenyl ist insofern problematisch, als die geringe vorhandene Menge (0.1 γ) meist schon vor der Sichtbarmachung wegsublimiert.

3. Diskussion der Tabelle II

Die deutlichste und nuancenreichste Anfärbung wurde durch Besprühen mit einer Lösung von *0.3 %igem Cer(IV)-sulfat in konzentrierter Salpetersäure* erzielt. Interessanterweise sind die mit Cersulfat behandelten Chromatogramme im kurzwelligen U.V.-Licht (254 mμ) erheblich schlechter erkennbar, wohingegen bei dieser Wellenlänge auf einer unbesprühten Platte alle Polyphenyle deutlich fluoreszieren (*o*-Terphenyl fluoresziert schwächer als der Untergrund; dunkler Fleck). Mit diesem Anfärbereagenz haben alle Stoffe, die ausschliesslich *meta*-Struktur besitzen die gleiche intensiv rotviolette Farbe; sie ergeben die erwarteten Mischfarben, wenn noch andere Bindungsarten vorliegen.

Cer-Salze in schwach salpetersaurer Lösung wurden schon von DUKE UND SMITH[11] zum Nachweis von sauerstoffhaltigen organischen Verbindungen benutzt (Rotfärbung); in stark schwefelsaurer Lösung reagieren sie auch mit Harnstoffderivaten zu farbigen Lösungen[12]. Schliesslich dienen sie zur Sichtbarmachung von jodhaltigen Verbindungen. Keine der anderen untersuchten Nachweismethoden hatte eine Empfindlichkeit, die unter 1 γ lag (mit Ausnahme der Fluoreszenz im kurzwelligen U.V.-Licht).

4. Identifizierung

(*a*) *R_{St}-Wert.* Wie später noch erwähnt wird, sind die Laufstrecken der Polyphenyle bei der DSC sehr stark von einer Reihe von Parametern abhängig, sodass oft die Angabe der R_F* fragwürdig wird. Wir hatten deshalb zunächst die Laufstrecken der Substanzen auf einen *inneren Standard* bezogen (R_{St}), um von einer Reihe von Zufälligkeiten unabhängiger zu werden** und *o*-Terphenyl gewählt:

$$R_{St} = \frac{\text{Laufstrecke Substanz}}{\text{Laufstrecke } o\text{-Terphenyl}}$$

(*b*) *Identifizierung durch Fluoreszenz.* Bei den höheren Polyphenylen, insbesondere bei praktischen Gemischen, folgen die Flecke oft sehr eng aufeinander, sodass die Identifizierung mit Hilfe des R_{St} und von Farbreaktionen schwierig wird. In vielen Fällen konnten wir diese dennoch durch Registrierung des Fluoreszenzspektrums vornehmen. Die gesamte Platte wird dazu, ohne dass die Substanzen ausgekratzt wurden, direkt in den Probenraum der Fluoreszenz-Anbaueinheit zum Cary 14-Spektralphotometer gebracht. In Fig. 1 sind die Fluoreszenz-Emissionsmaxima einer Reihe von synthetisierten Polyphenylen wiedergegeben***. Kurvenform und

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* Nach einem Vorschlag von BRENNER und Mitarbeiter[13] verwenden wir für den Ausdruck R_F-Wert „das R_F" (Singular) und „die R_F" (Plural), dementsprechend: „das R_{St}" und „die R_{St}".

** Verfälschungen durch querlaufende Inhomogenitäten der Schicht und Randeffekte lassen sich dadurch nicht eliminieren. Man muss dabei auch in Kauf nehmen, dass $R_{St} > 1$ auftreten können, z.B. bei Biphenyl.

*** Messungen von S. SANDRONI.

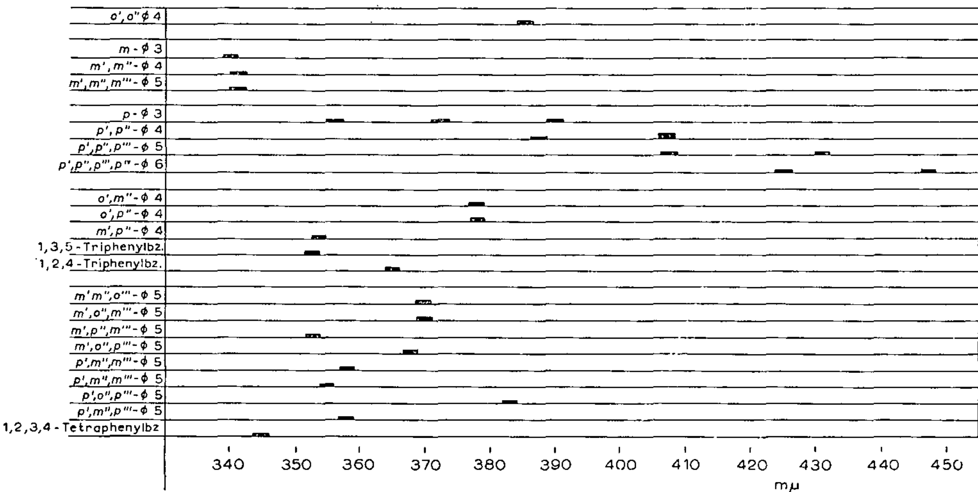

Fig. 1. Fluorenszenz-Emissions-Maxima ausgewählter Polyphenyle in Aluminiumoxid-Matrix. Erregerlicht: Quecksilberlinie 253 mμ.

Emissionsmaxima können von Gerät zu Gerät schwanken, sodass zur sicheren Identifizierung das Mitlaufen einer Vergleichsprobe angezeigt ist.

C. EINFLÜSSE AUF DIE AKTIVITÄT VON ALUMINIUMOXIDSCHICHTEN

1. Allgemeines

In einem vorläufigen Bericht[9] und in einem Vortrag, den einer von uns (F.G.) kürzlich gehalten hat[14], wiesen wir bereits auf stark variierende Trennresultate hin. Da wir dafür schwer zu kontrollierende Parameter wie Temperatur, Kammersättigung, Korngrössenverteilung, Ungleichheiten der Schicht und Struktur der Oberfläche verantwortlich machten, glaubten wir für das vorliegende Trennproblem an der Grenze der Leistungsfähigkeit der Methode angekommen zu sein.

Bei den vorgenannten Arbeiten hatten wir auch festgestellt, dass die Entwicklung auf einer heissen Platte meist günstigere Trennresultate brachte, als die auf einer kalten. Systematische Studien zeigten jedoch, dass diese erhöhte Trennleistung primär nicht durch einen *Temperatureffekt* zu erklären war (siehe Abschnitt C.3).

Fig. 2a zeigt schematisch den Verlauf dreier Chromatogramme desselben Testgemisches, in einer doppelwandigen Kammer isotherm bei 20°, 40° und 60° entwickelt. Das Gesamtbild der drei Trennungen ist ähnlich; mit zunehmender Temperatur verkürzt (!) sich die Laufstrecke der Höhermolekularen. Eine Erklärung dafür wird im Abschnitt C.2 gegeben.

Zwei weitere Experimente zum Studium des Einflusses der Temperatur wurden in einem Klimaschrank bei konstanter relativer Feuchte (nachfolgend „r.F." abgekürzt) (45%, Fig. 2b, 65%, Fig. 2c) ausgeführt. Hier sind die praktischen Trennresultate einander noch ähnlicher, obwohl die Laufstrecken jetzt mit zunehmender Temperatur grösser geworden sind, d.h., dass die R_{St} im Wesentlichen gleich geblieben sind. Auch darauf wird unter C.2 noch näher eingegangen.

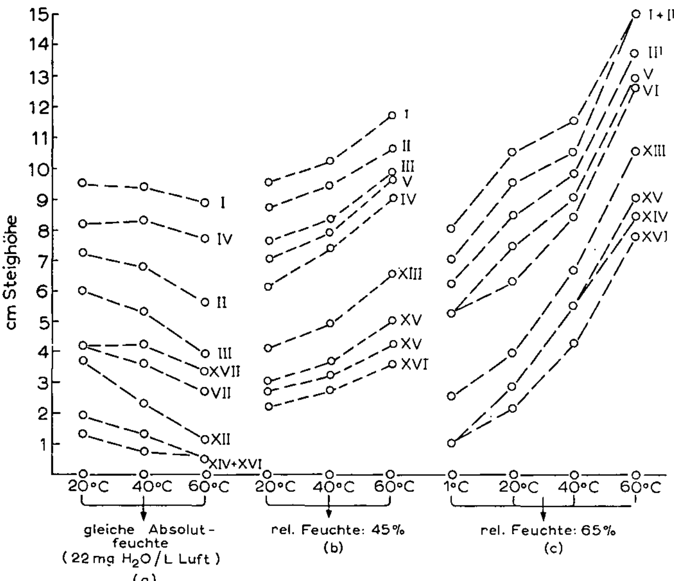

Fig. 2. Einfluss von Temperatur und Feuchte auf die DSC-Trennung von Polyphenylgemischen (schematisch). (a) Einfluss der Temperatur bei gleichbleibender absoluter Raumfeuchte. Die durch die Temperaturerhöhung verringerte Aktivität wird hinsichtlich der Laufstrecken durch den Effekt der abnehmenden relativen Feuchte kompensiert. (b) und (c) Die relative Feuchte bleibt konstant; mit zunehmender Temperatur verringert sich die Aktivität: Die Laufstrecken werden grösser. Sorbens: Al_2O_3, Merck; Eluens: *n*-Heptan.

2. *Einfluss der Luftfeuchte**

Die ursprüngliche Annahme, dass die Nichtreproduzierbarkeit der Trennung auf Fabrikationsschwankungen des Aluminiumoxids beruhe, war nicht mehr haltbar, als sich herausstellte, dass dasselbe Schichtmaterial an zwei Orten verschiedenen Klimas einmal etwa 10 Verbindungen aufgetrennt hatte, einmal unter sonst gleichen Bedingungen praktisch keine Trennung mehr zustande brachte. Damit blieb als entscheidender Parameter noch die Feuchtigkeit übrig. Es zeigte sich, dass die Trennung eines Testgemisches, das 9 Stoffe (von *o*-Terphenyl bis zu Hexaphenylen) enthielt, völlig zusammenbrach, wenn die relative Feuchte während Vorbereitung und Elution ca. 70% überstieg. Die Zusammenhänge von Adsorptions-Aktivität** und Luftfeuchte wurden durch folgende Versuchsreihe klar (Fig. 3):

Sieben gleiche, „klassisch" vorbehandelte Aluminiumoxidplatten wurden einzeln in Klimakammern während 20 Stunden bei Zimmertemperatur relativen Feuchten von 1–82% ausgesetzt und dann in einer Kammer bei der jeweils gleichen Feuchte entwickelt. Das Ergebnis ist eindeutig: bei 1% r.F. hat sich nur das *o*-Terphenyl deutlich vom Startpunkt abgehoben ($R_F = 0.3$). Mit zunehmender Feuchte lösen sich auch die höhersiedenden Polyphenyle (nachfolgend kurz „HS" genannt) vom Startpunkt ab, bis sich zwischen 37 und 50% r.F. ein Trennoptimum einstellt.

Jenseits dieser Grenze werden die Chromatogramme wieder unbrauchbar. Bei

* Alle in diesem Kapitel beschriebenen Chromatogramme wurden mit *n*-Heptan als Laufmittel ausgeführt.
** Im BROCKMANN'SCHEN[15] Sinne die Grösse einer „Intensitäts-Bindungsstärke".

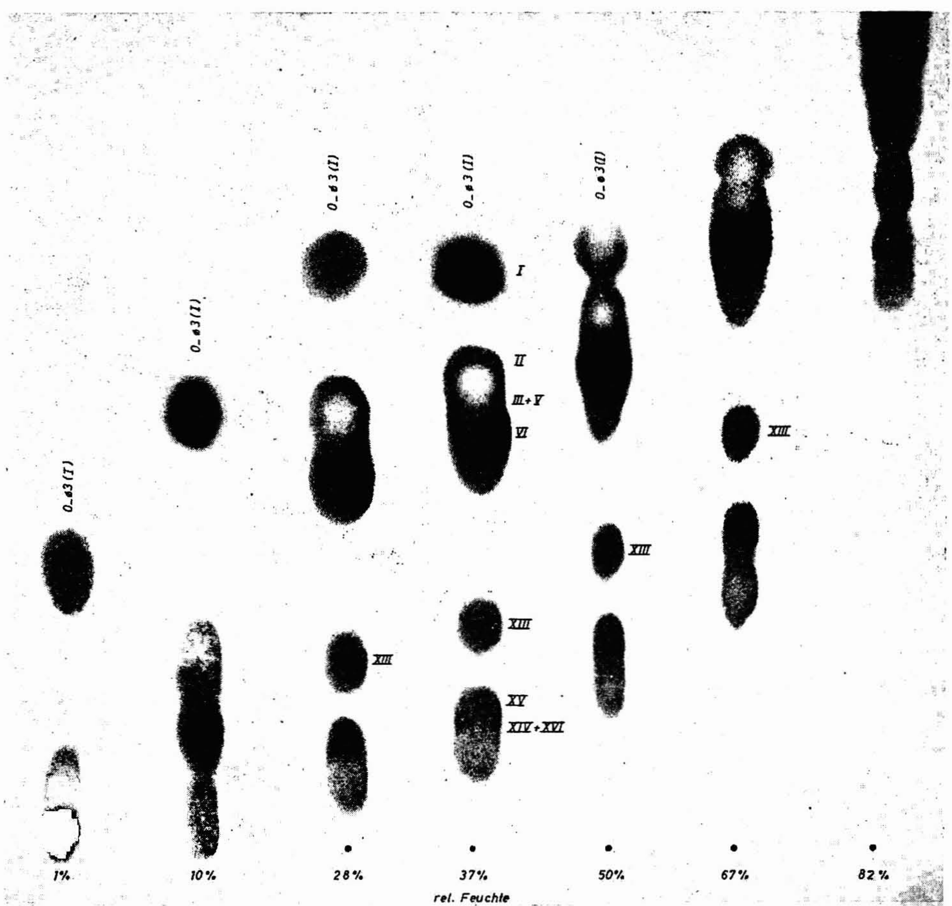

Fig. 3. Isotherme Trennung eines Polyphenyltestgemisches bei verschiedenen Klimata (Photomontage). Aufgetragene Menge/Startpunkt 0.5 γ insgesamt. Eluens: *n*-Heptan; Sorbens: Al_2O_3, Merck; Sichtbarmachung: Besprühen mit 0.2 %iger $Ce(SO_4)_2$-Lösung in konz. HNO_3; Photographie im U.V.-Licht 366 mμ. Die Platten wurden 24 Stunden vorklimatisiert und bei der jeweils gleichen Feuchte auch eluiert.

82 % r.F. befinden sich praktisch alle Substanzen zusammen an der Front. Am Start bleiben jeweils geringe Mengen stark fluoreszierender Verunreinigungen zurück, die von der Synthese herstammen. In dieser Reihe ist das Verhalten des *o*-Terphenyls am auffälligsten: Zwischen 28 und 50 % r.F. scheint sich ein reproduzierbares „Plateau" auszubilden. CORNELIUS und Mitarb.[16] untersuchten die Wasseraufnahme von bei 538° kalziniertem Aluminiumoxid als Funktion der relativen Feuchte und fanden nach einem steilen Anstieg zwischen 0 und 10 % r.F. ebenfalls eine Art „Plateau" zwischen 10 und 50 % relativer Feuchte. Jenseits dieses Wertes stieg die Wasseraufnahme wieder stark an. Im Bereich bis zu 10 % r.F. postulieren diese Autoren die Ausbildung einer monomolekularen Schicht, bei höheren Feuchten sollen sich Mehrfachschichten bilden*.

* Zu ähnlichen Resultaten kamen DE BOER und Mitarb.[17] in ihrer Studie über den Aufbau „aktiver" Aluminiumoxide. Bei 15 % r.F. soll sich schon die erste Schicht physikalisch gebundenen Wassers aufgebaut haben. Oberhalb 50 % r.F. soll dann die Kapillarkondensation in den Poren einsetzen.

Die von CORNELIUS und Mitarb.[16] beschriebene Wassersorption war zwischen 30° und 500° reversibel. Die in Fig. 3 wiedergegebenen Effekte sind innerhalb des Messfehlers reproduzierbar, die Feuchte–Aktivitäts-Abhängigkeit ist ebenfalls reversibel. Zwei Platten a und b wurden 1 Stunde bei 105° getrocknet, über Blaugel abgekühlt, mit dem Testgemisch versehen und anschliessend 6 Stunden bei 73 % r.F. klimatisiert. Hernach wurde Platte a bei 73 % r.F. entwickelt, während b einem Klima von 7.5 % r.F. ausgesetzt und anschliessend dort entwickelt wurde. Zusätzlich wurden zwei Platten c und d, wie a und b getrocknet und bei 7.5 % r.F. klimatisiert. Platte c wurde unmittelbar bei dieser Feuchte entwickelt, d erst nach „Umklimatisierung" bei 73 % r.F. bei jenem Klima. Die Platten a und d einerseits und b und c andererseits zeigen kongruente Trennungen.

Mit den neuen Erkenntnissen sind nun auch die *Temperatureffekte* der Versuche (Fig. 2, a–c) zu verstehen:

(a) Bei Fig. 2a ist die relative Feuchte mit steigender Temperatur abgesunken (die absolute Feuchte blieb gleich). Dies hätte, allein betrachtet, eine Zunahme der Aktivität und eine Verkürzung der Laufstrecken zur Folge. Gleichzeitig setzt aber eine Temperaturerhöhung die Aktivität notwendigerweise herab, die Laufstrecken müssten also grösser werden. Diese beiden Effekte kompensieren sich (insbesondere für die leichteren Moleküle) weitgehend; lediglich die HS zeigen die für niedrige Feuchten typische Tendenz, in der Nähe des Startpunktes zusammengeballt zurückzubleiben, d.h. *die R_F sind eine Funktion der relativen Feuchte.*

(b) Bei Fig. 2b und Fig. 2c wurde ebenfalls die Temperatur erhöht, die relative Feuchte aber konstant gehalten (d.h. die absolute Feuchte erhöhte sich). Jetzt sind die nur feuchtigkeitsabhängigen R_{St} konstant geblieben, der durch die Temperaturerhöhung bedingte Aktivitätsabfall der Platte verschob die R_F jedoch gleichförmig zu höheren Werten.

Bei vorausgegangenen orientierenden Experimenten hatten wir versucht, den *Einfluss der Plattenpräparierung und „-aktivierung"* zu klären. Die Ergebnisse erschienen zunächst kurios:

(a) Zwei Platten, von denen eine 1 Stunde an der Luft getrocknet worden war, die andere 1 Stunde bei 105° im Trockenschrank, zeigten die gleiche Aktivität, wohingegen oft

(b) zwischen bei 105° getrockneten Platten der gleichen Streich-Charge bei Entwicklungen an verschiedenen Tagen oder Stunden enorme Unterschiede bestanden.

(c) Selbst Platten, die bei 105° aktiviert, mit den Substanzen bereits aufgetragen über konzentrierter Schwefelsäure aufbewahrt wurden, gaben während eines längeren Zeitraumes keine Aktivtrennung.

(d) Sechs Platten wurden bei sechs verschiedenen Feuchten klimatisiert (Tabelle III) und dann alle bei Raumfeuchte (die bei 70 % lag) entwickelt. Die Platte mit 91 % r.F. bringt „ihr Klima" mit, das von der niedereren Luftfeuchte während der kurzen Zeit der Entwicklung nicht mehr wesentlich beeinflusst wird; alle anderen Platten nehmen die dem Raumklima entsprechende Aktivität an.

(e) Als Kontrollexperiment wurden vier Platten mit dem Testgemisch, die verschieden vorbehandelt worden waren, ohne vorherige Klimatisierung bei 10 % r.F. entwickelt. Die eine Stunde an der Luft getrocknete Platte lieferte nur einen amorphen Fleck an der Front, die zweite, die drei Stunden an der Luft gelegen hatte, zeigte eine

TABELLE III

AUFHEBUNG DER VORKLIMATISIERUNG VON DSC-PLATTEN (Al_2O_3) BEIM AUFTRAGEN UND ENTWICKELN BEI RAUMKLIMA

Die Platten mit *p*-Terphenyl (III) wurden bei verschiedenen Feuchten (1. Spalte) klimatisiert und dann bei Raumklima (70 % relative Feuchte) entwickelt. Nur die feuchteste Platte behält „ihr" Klima, die anderen werden nivelliert.

Rel. Feuchte (%)	*Temp. (°C)*	*Einstellzeit (Stunden)*	R_F *von p-ϕ3*
21	26	24	0.48
32	25	72	0.46
51	25	24	0.45
63	26	24	0.50
75	25	27	0.48
91	24	18	0.86

Andeutung einer Trennung an der Front*; die bei 105° und 220° vorbehandelten Platten lieferten identische Trennungen, wie sie etwa nach Vorklimatisierung bei 60° erhalten worden wären (siehe Fig. 3).

Aus diesen und aus den vorstehenden Versuchen lässt sich auch entnehmen, dass die Wassersorption an Aluminiumoxid erheblich schneller vonstatten geht als die Desorption**. Dies ist in der Praxis der Plattenvorbereitung insbesondere hinsichtlich der während des Auftragens der Substanzen verstreichenden Zeit von Bedeutung.

(f) Die Gewichtszunahme einer Platte mit Aluminiumoxid, die bei 105° vorgetrocknet war und an der Luft (65 % r.F.) abkühlte, betrug bei 2 mm Schichtdicke nach 6 Min. schon 2 %, nach 20 Min. über 3 %. Wie Fig. 4 zeigt, ist die prozentuale Wasseraufnahme deutlich von der Schichtdicke abhängig. Die Wasseraufnahme einer

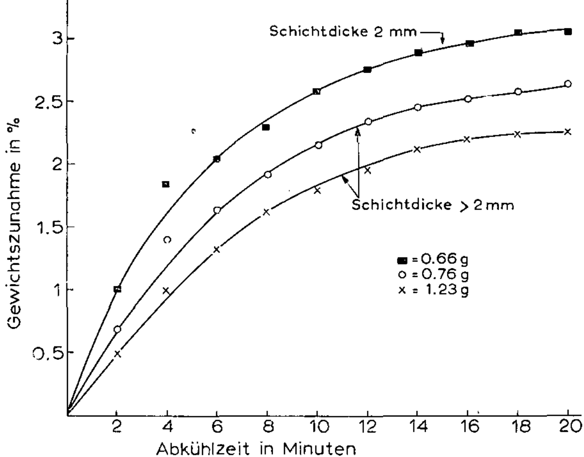

Fig. 4. Wasseraufnahme von Al_2O_3-Schichten während des Abkühlens von 105° auf 20°. Schicht bei 0.76 g und 1.23 g mit dem Spatel, bei 0.66 g mit einem Streichgerät bestrichen. Raumfeuchte: 65 %.

* Dieses Resultat steht nicht im Widerspruch zu dem des Versuchs (a): In diesem Falle betrug die Raumfeuchte 65 % r.F., in jenem nur 40 %.

** Eine echte Hysterese scheint jedoch nicht vorzuliegen.

normal dicken Schicht (0.25 mm) dürfte also noch wesentlich höher sein, als die der 2 mm dicken Schicht. Diese Nivellierung der Aktivität der Platten auf die gerade herrschende Raumfeuchte hin wird auch durch die Tabelle III verdeutlicht.

Fig. 5 demonstriert die Wasserde- und -absorption von Aluminiumoxidproben, die, ausgehend von einer Gleichgewichtsfeuchte bei 20% r.F. — willkürlich als

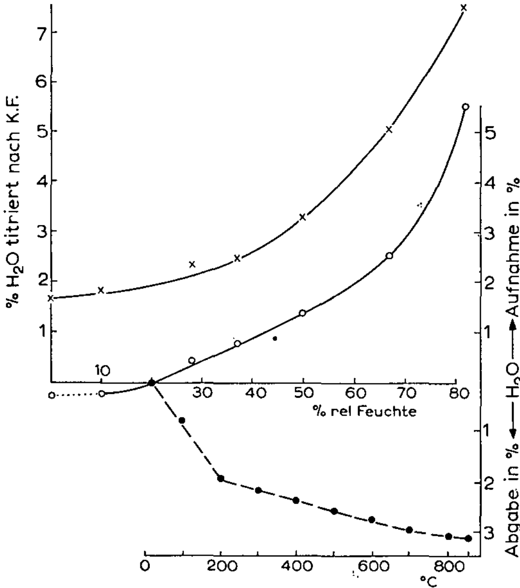

Fig. 5. Wasserhaushalt von Al_2O_3-Schichten für DSC. (Der darin enthaltene Gips ist totgebrannt.) *Oberer Teil*: Isotherme Wasseraufnahme (bzw. Abnahme) in Abhängigkeit des Klimas. Linke Kurve: Wassertitration nach der Karl-Fischer-Methode (linke Ordinate). Rechte Kurve: Gewichtsmässige Wasserab- bzw. -desorption. Der Nullpunkt ist willkürlich bei 20% Gleichgewichtsfeuchte gewählt. *Unterer Teil*: Thermogravimetrische Messung der Wasserabspaltung bis 850° (Probe bei 20% relativer Feuchte vorklimatisiert.)

Bezugspunkt 0% angenommen — parallel zu Versuchen der Fig. 3 jeweils 24 Stunden verschiedenen Klimata ausgesetzt wurden.

Dass die Kurve „mit Karl-Fischer-Reagenz titrierbarer Wassergehalt" der Kurve „Gewichtszunahme" praktisch parallel läuft, ist ein weiterer Beweis dafür, dass das aufgenommene Wasser nur schwach, physikalisch, gebunden sein kann. Aus Fig. 4 und 5 lässt sich weiter entnehmen, dass eine Platte, die bei 105° „klassisch" aktiviert wurde, nach einer Abkühlzeit von 5 Min. (der Zeit, die normalerweise zum Auftragen des Substanzen benötigt wird), schon 1.5% Wasser, bezogen auf das Schichtmaterial oder 70% des Gleichgewichtswassergehaltes bei 55% Raumfeuchte aufgenommen hat. Das Trennergebnis unseres Polyphenyl-Testgemisches ist darum dann nicht mehr das einer Aktivplatte, sondern entspricht etwa dem einer Gleichgewichtsklimatisierung bei 45% relativer Feuchte. Bei noch höheren Feuchten steigt diese Absorptionsgeschwindigkeit für Wasser exponentiell an.

Der Gasraum in einer klassischen Desaga-Kammer beträgt ca. 3500 cm^3, in einer „S-Kammer"* ca. 100 cm^3. Mit Luft von 72% r.F. gefüllt, entspricht dies einer

* Siehe Praktischer Teil, Abschnitt E.2.

Wassermenge von 80 mg bzw. 2.4 mg[25] (Verhältnis 35:1). In diese beiden wurde je eine mit Blaugel (ca. 20 % r.F.) vorgetrocknete Platte direkt eingestellt und mit *n*-Hexan eluiert. *o*-Terphenyl liegt bei beiden dann wieder auf der gleichen Höhe, während die Differenzen für die anderen R_F beträchtlich sind. Wie zu erwarten, verlief die Trennung in der Normalkammer viel „feuchter" als in der anderen. Die Chromatogramme entsprechen etwa denen einer Entwicklung bei 50 % bzw. 28 % *Gleichgewichtsfeuchte* (siehe Fig. 3). Wurden hingegen die beiden Platten bei derselben relativen Feuchte, bei der sie auch entwickelt wurden, vorklimatisiert, so blieb die Art der Kammer ohne Einfluss.

Auf Grund dieser quantitativen Ergebnisse lässt sich jetzt auch schlüssig erklären, warum oberhalb einer bestimmten Raumfeuchte, praktisch unabhängig von der Aktivierungstemperatur, eine hohe Aktivität *dann nicht* mehr erreicht wird, wenn die Platten auch nur einige Minuten der Raumluft ausgesetzt gewesen sind. Wie dieses Handicap des Aluminiumoxidadsorbens* in vielen Fällen umgangen werden kann, wird im Abschnitt C.3 „Heisselution" ausgeführt.

Oberflächenbedeckung. Die der Fig. 6 (oberer Teil) zugrunde liegende Probe Aluminiumoxid G wog 3.8 g, dem entspricht bei einem Gipsgehalt von 15 % 3.2 g Aluminiumoxid. Nach Herstellerangabe[18] besitzen Aluminiumoxide für die Chromatographie spezifische Oberflächen von ca. 150 m^2/g**. 3.2 g besitzen dann 480 m^2 Oberfläche. Wenn man annimmt, dass ein Wassermolekül 10.8 $Å^2$ beansprucht, so haben darauf $4.4 \cdot 10^{21}$ Moleküle oder 0.13 g Wasser in einer monomolekularen Schicht Platz; das sind ca. 3.7 % bezogen auf 3.8 g Aluminiumoxid G mit dem darin enthaltenen Gips als Hemihydrat, oder: bei der Annahme einer Ungenauigkeit der spezifischen Oberfläche von $\pm$ 20 % bauen sich oberhalb eines Bereiches von 3.0–4 % Gewichtszunahme (bei Bezug auf 1 % r.F. entsprechend oberhalb 73–78 % Gleichgewichtsfeuchte, siehe Fig. 6) *Mehrfachschichten* auf***.

Dieser Grenzwert beruht auf einer Überschlagsrechnung. Weitere Unsicherheitsfaktoren sind die Messwerte der relativen Feuchte (Haarhygrometer) und nicht zuletzt der schwer kontrollierbare Einfluss der Gipsbeimischung. Die errechneten Werte befinden sich dennoch in guter Übereinstimmung mit den Resultaten in Fig. 3. Bei 82 % r.F. ist die Adsorptionschromatographie praktisch in eine Verteilungschromatographie übergegangen; entsprechend den Löslichkeiten liegt das Verteilungsgleichgewicht praktisch auf der Seite der organischen Phase§.

Diese Ergebnisse erlauben wahrscheinlich auch noch folgenden Schluss: Mit fortschreitender Wasseraufnahme wird nicht etwa eine bestimmte Anzahl *gleichartiger* aktiver Zentren sukzessive besetzt, sondern es müssen zuerst aktivere Plätze bevorzugt belegt werden, wenn man von der Beobachtung ausgeht, dass dann nicht eine Parallelverschiebung der Laufstrecken stattfindet, was auf ein allmähliches Verschwinden gleichartiger Adsorptionsstellen schliessen liesse, sondern eine Diskriminierung bestimmter R_{St} mit der wechselnden Feuchte.

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* N.B. Aluminiumoxid „Fluka" zur DSC besitzt gegenüber dem Merck'schen Produkt eine etwas erhöhte Grundaktivität, doch sind die Veränderungstendenzen bei Feuchtigkeitseinfluss gleichförmig.

** Bestimmt nach der BET-Methode mit flüssigem Stickstoff.

*** Wenn solche nicht vorher schon existierten.

§ Bei Erhitzen einer solchen bei 25 % vorklimatisierten Aluminiumoxidprobe bis 850° auf einer Thermowaage (Raumklima 40 %), gibt diese noch einmal 3.1 % Wasser ab, das wohl in den verschiedenen möglichen Hydratformen, grösstenteils chemisch gebunden, vorliegt (Fig. 5 unten).

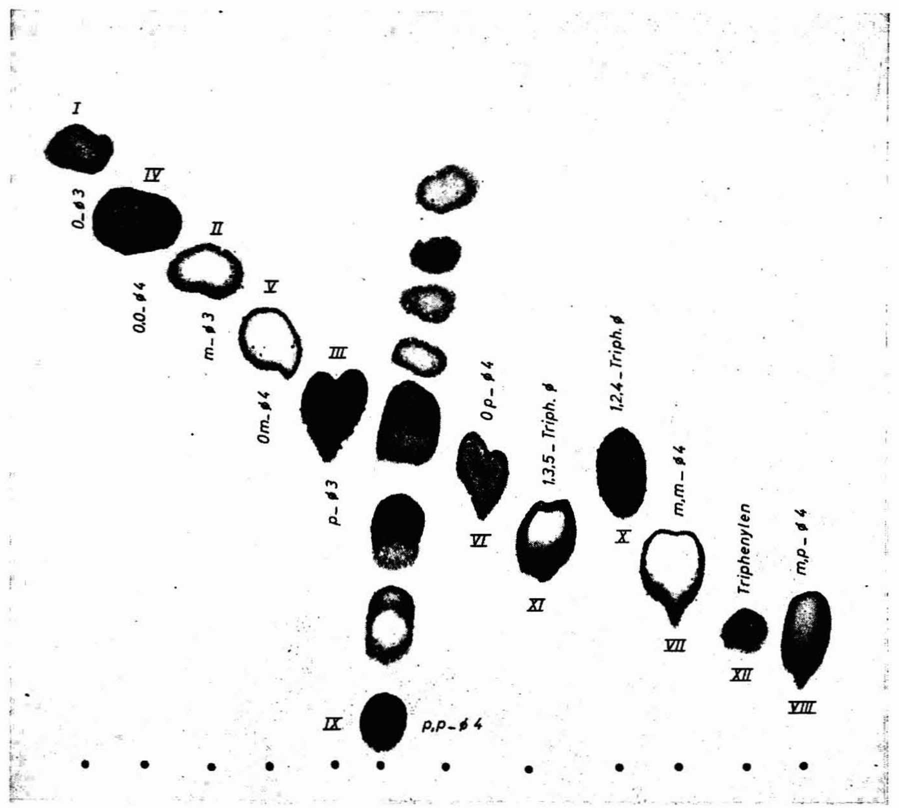

Fig. 6. Trennung der Quaterphenyle mit „Heisselution". Relative Feuchte: 40 %. Sonstige Daten wie bei Fig. 3.

Es werden bei einigen Stoffpaaren sogar Inversionen beobachtet. So haben z.B. die Substanzen: (1) *p*-Terphenyl (III), (2) *o,p*-Quaterphenyl (VI) und (3) *o,m*-Quaterphenyl (V) nach zunehmendem R_F geordnet bei 5 % r.F. die Reihenfolge: 1, 2, 3, bei 15 % r.F.: 2, 1, 3 und bei 45 % r.F.: 2, 3, 1. Untersuchungen über Zusammenhang dieser Diskriminierung und dem sterischen Bau dieser Substanzen sind im Gange.

3. „Heisselution"

Werden DSC-Platten mit Polyphenylen noch warm (ca. 40°) in die Entwicklungskammer gebracht, so ist meistens die Aktivität höher und auch die Trennung der Substanzen besser, als bei kalten Platten. Die Trennung durchläuft für unser Problem ein Optimum bei 40 % r.F. in der Trennkammer. Bei der Feuchte um 80 % ist die Trennung dementsprechend nur noch schwach.

Bei dieser „Heisselution" überlagern sich zwei Effekte:

(a) Während des Hantierens der heissen Platte an der Luft schlägt sich weniger Feuchtigkeit nieder, als dies bei einer kalten aus dem Exsikkator kommenden geschähe. Während der weiteren Abkühlung in der Trennkammer setzt sich das Schichtmaterial erst allmählich ins Gleichgewicht mit dem der Kammerfeuchte.

(b) Durch die erhöhte Temperatur tritt auf der Platte auch stärkere Verdampfung

des Eluens ein, d.h. die Wanderungsgeschwindigkeit der Front wird verlangsamt, der Fliessmitteldurchsatz aber erhöht (siehe auch JAKUBEK[19]). Das Resultat ist dann eine stärkere Elution und damit ein Höherwandern der Flecken, ohne dass damit die Aktivität geändert wurde.

Die Wirkung dieser „Heisselution" in der klassischen Entwicklungskammer, in der der Dampfraum noch viel Lösungsmittel aufnehmen kann, ist deutlich stärker als in der sogenannten „S-Kammer" der Desaga (siehe Praktischer Teil), bei der der Raum zwischen Schicht und der darauf aufliegenden Deckplatte schnell gesättigt ist. Die Wanderungsgeschwindigkeit der Front in dieser Kammer ist erheblich grösser, als die in der klassischen Kammer.

In Fig. 6 ist die Trennung von acht isomeren Quaterphenylen zusammen mit den Terphenylen wiedergegeben (durch Heisselution). Keine der vorerwähnten Temperatur–Feuchtepaare ermöglichte eine so weitgehende Trennung. Weitere Chromatogramme mit der Trennung technischer Polyphenylgemische sind an anderer Stelle[9, 20] veröffentlicht.

4. Zusammenfassung der Resultate auf Aluminiumoxid

(a) Durch Vorklimatisierung der Platten und späterer Entwicklung bei gleicher Feuchte können beliebige Aktivitäten eingestellt und entsprechende Trennergebnisse erzielt werden.

(b) Bei Polyphenylgemischen steht den leichteren Verbindungen bei niederer Feuchte praktisch der gesamte Raum zwischen Start und Front zur Verfügung, da die HS am Start zurückbleiben. Umgekehrt, wird der Bereich der Quinqua- und Sexiphenyle gut aufgetrennt, wenn mittlere Feuchten eingestellt wurden, während sich die Terphenyle, nur knapp aufgetrennt, in der Nähe der Front befinden.

(c) Steht ein Klimaschrank zur Verfügung, so ist es möglich, für ein Trennproblem die jeweils günstigste Temperatur–Feuchtekombination auszusuchen*.

(d) Dem Ziel, konstante R_F auch bei der DSC zu erreichen, rückt man näher, wenn die hydrothermalen Bedingungen kontrolliert werden können. Dazu müssen allerdings Sorbentien ähnlicher Grundaktivität, gleichartige Lösungsmittel und eventuell Entwicklungskammern eingesetzt werden. Um insbesondere Herstellungsschwankungen zu eliminieren, schlagen BRENNER und Mitarb.[13] vor, die einzelnen Chargen durch theoretisch begründete Konstanten zu sichern.

(e) Inwieweit die bei der Trennung der Polyphenyle beobachtete *starke* Abhängigkeit der R_F und R_{St} von den klimatischen Bedingungen auf diese hydrophobe Stoffklasse beschränkt bleibt, muss noch untersucht werden.

5. Elution mit Lösungsmittelgemischen

Das schon mehrmals, insbesondere beim *o*-Terphenyl beobachtete Plateau scheint sich auch auszubilden, wenn man die hydrothermalen Bedingungen (20 % r.F.) konstant hält und dem Fliessmittel *n*-Heptan sukzessiv Benzol zusetzt (Fig. 7). Hier ersetzen offenbar die Benzolmoleküle bevorzugt (an Stelle der oben postulierten Wassermoleküle) die aktive Sorbensoberfläche.

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* SCOTT[21] beeinflusste mit der Feuchteregulierung einer gaschromatographischen Aluminiumoxidsäule die relativen Rückhaltewerte gesättigter und ungesättigter gasförmiger Kohlenwasserstoffe.

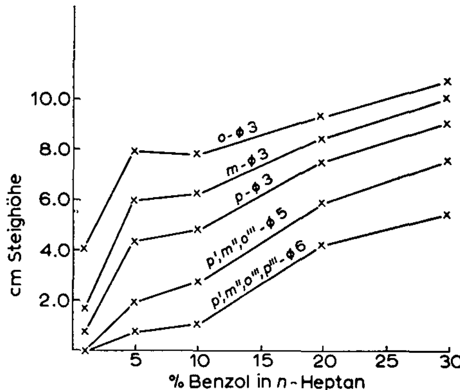

Fig. 7. Einfluss der Benzolkonzentration im Eluens (Schematische Chromatogramme). Sorbens: Al_2O_3.

D. TRENNVERSUCHE MIT ANDEREN SORBENTIEN

1. Fluoreszierende Schichten

Fluoreszierende Schichten (Al_2O_3) bringen keinen Vorteil hinsichtlich Erkennbarkeit der aufgebrachten Substanzen.

2. Nylon

Nylon als Schichtmaterial (mit Gipszusatz als Haftmittel) ermöglicht mit Aceton–Wasser (2:1) als Fliessmittel eine Trennung der drei Terphenyle untereinander und von den HS, die allerdings selbst nicht unterschieden werden. Die Nylonschicht erschwert die Sichtbarmachung der Substanzen, weil sie selbst stark fluoresziert.

3. Magnesiumsilikat

Magnesiumsilikat („Florosil®")* mit Gipszusatz und Cyclohexan als Laufmittel ermöglicht eine mässige Trennung der Höhersiedenden. Eventuell bringt auch hier eine Vorklimatisierung, die nicht näher untersucht wurde, dem Aluminiumoxid gleichwertige Trennresultate.

4. Kieselgel

Kieselgelschichten geben bei dem vorliegenden Trennproblem nur magere Ergebnisse. Es findet andeutungsweise eine Trennung nach Molekulargewicht statt. Mit Vorteil lassen sich diese Schichten jedoch zur Untersuchung von Verunreinigungen nicht polyphenylischer Natur in reinen Polyphenylen verwenden. Bei diesem Sorbens ist der Einfluss der Feuchte auf die Trennaktivität noch gravierender als bei Aluminiumoxid: Das Hexaphenyl (XVI) hat auf Kieselgel G (Merck) mit Tetrachlorkohlenstoff als Laufmittel bei 40 % ein R_F von 0.15, bei 65 % r.F. schon 0.72 (500 % Differenz!).

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* Floridin Comp., Tallahassee, Fla.

E. PRAKTISCHER TEIL

1. Herstellung der Platten

Die Herstellung der Platten wurde nach der klassischen Vorschrift von STAHL[22] vorgenommen.

2. Entwicklungskammern (nicht klimatisiert)

Ausser der in der Grundausrüstung Nr. 600 der Firma Desaga* enthaltenen Entwicklungskammer, wurde ein von STAHL entwickeltes und als „S-Kammer" bezeichnetes Gerät** benutzt: Eine beschichtete Platte wird über einen 2 mm dicken Abstandshalter gegen eine zweite leere Platte gestellt und zur Entwicklung in einen „Rohrtank", der das Laufmittel enthält, eingetaucht.

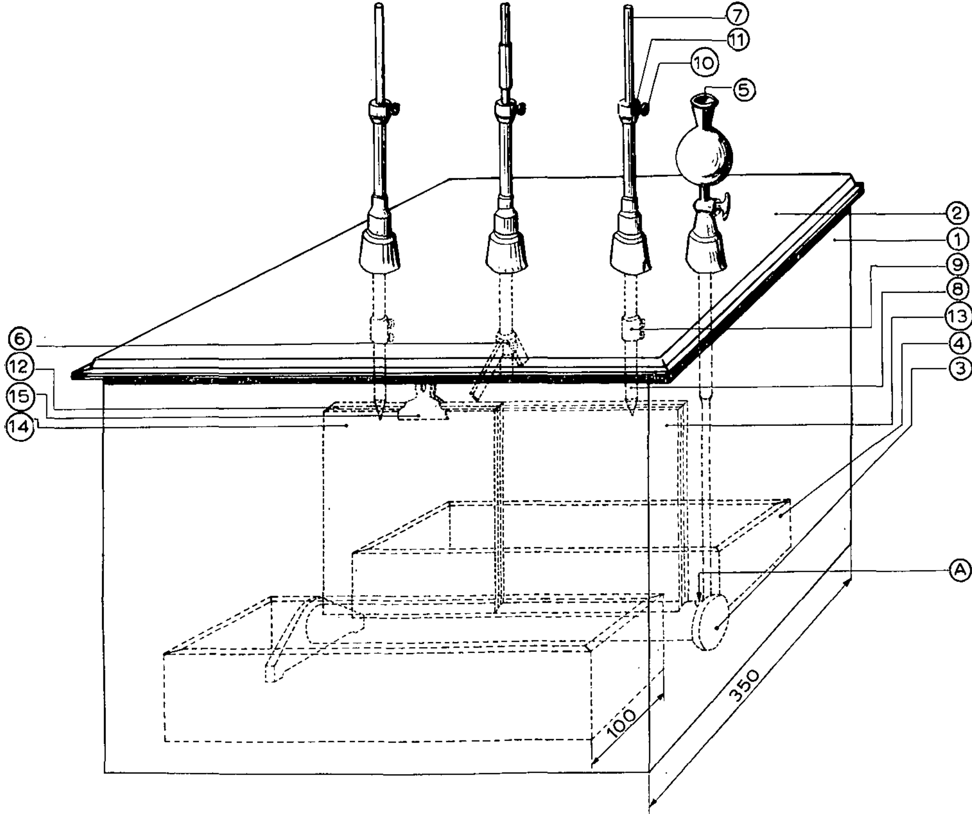

Fig. 8. Grosse Klimakammer zur Vorklimatisierung und Entwicklung von DSC-Platten. Handhabung siehe Praktischer Teil. 1 = Kammer aus Glas; 2 = Deckel mit 4 Bohrungen und 3 KPG-Hülsen; 3 = Rohrtank („Desaga") für 200 × 400 mm Platten mit zusätzlicher Bohrung A; 4 = Trog für Schwefelsäure; 5 = Tropftrichter mit NS 29; 6 = KPG-Rührwelle mit Polyäthylenflügeln; 7 = KPG-Welle; 8 = Teflonkeil; 9 = Teflonmuffe; 10 = Schraube aus Teflon zu 9 und 11; 11 = Haltering aus Teflon; 12 = Rahmen (Distanzstreifen); 13 = Rahmenplatte und Platte 200 × 400 mm; 14 = Rahmenplatte und Platte 200 × 200 mm; 15 = Metallklammer zum Zusammenhalten von Rahmen- und DSC-Platte; A = Zusätzliche Bohrung zum Einfüllen des Lösungsmittels.

* Heidelberg, Hauptstrasse 60.

** Vom gleichen Hersteller zu beziehen.

3. Klimatisierung und Entwicklung der Platten

(a) Die der Fig. 3 zugrundeliegenden Versuche wurden in folgender Kammer ausgeführt (siehe Fig. 8). In einem mit einem Deckel mit vier Bohrungen versehenen Glasgefäss wird die gewünschte Feuchte durch Aufstellen von Schwefelsäure verschiedener Dichte[23] eingestellt. Ein durch eine KPG-Hülse geführter und von einem Rührmotor angetriebener Wedel sorgt für gute Umwälzung der Atmosphäre. Die normal beschichteten und getrockneten Platten werden mit Substanzen versehen, mit der (leicht modifizierten) Rahmenplatte bedeckt und in den Rohrtank eingestellt. Diese gesamte „S-Kammer" wird dann in die Klimakammer eingebracht; danach werden Rahmenplatte und Adsorbensträger mit Hilfe einer von aussen zu bedienenden Vorrichtung mit einem Keil gespreizt, dass die klimatisierte Atmosphäre Zugang zum Adsorbens hat. Nach 24 Stunden (nachdem sich das Gleichgewicht sicher eingestellt hat) zieht man den Keil zurück, eine vorher aufgesetzte Klammer presst nun die

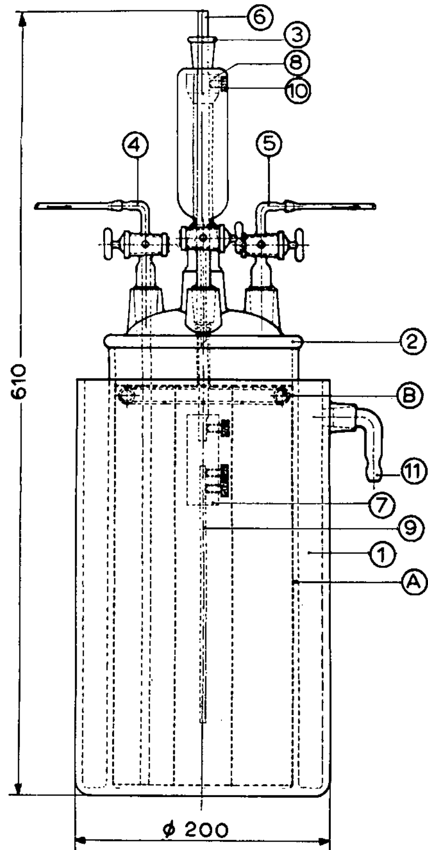

Fig. 9. Kleine Klimakammer. Handhabung siehe Praktischer Teil. 1 = Zylindrisches Entwicklungsgefäss aus Glas; 2 = Deckel mit 4 KPG-Hülsen; 3 = Tropftrichter; 4 = Gaseinleitungsrohr mit Hahn; 5 = Gasauslass mit Hahn; 6 = KPG-Welle; 7 = Muffe aus Teflon; 8 = Haltering aus Teflon; 9 = DSC-Platte; 10 = Schrauben aus Teflon zu 8; 11 = Überlauf für Thermostatflüssigkeit; A = Filterpapiereinlage an der Kammerinnenwand; B = Glasring mit Bohrung zur Verteilung des Eluens.

Platten zusammen. Das Laufmittel wird von aussen durch einen Tropftrichter in den Rohrtank eingefüllt. Die relative Feuchte wurde mit einem Haarhygrometer* bestimmt.

Andere Versuche wurden in kontrolliertem Klima mit der normalen eckigen Desaga-Kammer vorgenommen. In diesem Falle sind die Manipulationen jedoch schwieriger auszuführen und beeinflussen den Ablauf der Versuche.

(b) Klimatisierung durch Spülen des Gasraumes. Um die zum Teil recht langen Klimatisierungszeiten zu verkürzen, wurde ein Teil der beschriebenen Trennungen in einem weiteren Kammertyp ausgeführt (Fig. 9). Eine von der Firma Jenaer Glaswerke, Schott & Gen. hergestellte zylindrische Entwicklungskammer für die Papierchromatographie wird mit Filterpapier „ausgeschlagen". Durch die mittlere Bohrung im Deckel des Gefässes wird ein KPG-Stab eingeführt, an dem die präparierte Platte über eine Teflonmuffe in der Weise befestigt wird, dass sie den Boden des Gefässes zunächst noch nicht berührt. Durch seitliche Stutzen wird nun solange trockener Stickstoff durchgeblasen, bis der an einem Hygrometer abzulesende gewünschte Feuchtegrad erreicht ist. Durch eine weitere Bohrung wird das Eluens auf einen ringförmigen Verteiler (die Kammerwand benetzend) zugegeben, so dass das Filterpapier gleichmässig befeuchtet wird. Nach 5 Min. wird dann die Platte eingetaucht.

(c) Die Versuche der Fig. 2b und 2c wurden in einem Klimaprüfschrank**, regelbar von 10 % bis 95 % relativer Feuchte und von —70° bis +100° ausgeführt.

4. „Heisselution"

Die als "Heisselution" bezeichneten Trennungen fanden auf Platten statt, deren Temperatur höher als die des Lösungsmittels und die der Kammer war. Die Platten wurden vor der Elution auf 100° erwärmt und auf die für die jeweilige Trennung optimale Temperatur (meist ca. 40°) abkühlen lassen.

5. Adsorptionsmittel

Als Adsorptionsmittel wurde mit Ausnahme der in Abschnitt D.4 aufgeführten Trennungen Aluminiumoxid G zur Dünnschichtchromatographie nach STAHL der Firma Merck, Chargennummer 6356 verwendet.

6. Wasseraufnahme durch Aluminiumoxid

Eine Probe Aluminiumoxid G wurde 10 Stunden bei 350° geglüht, um den darin enthaltenen Gips „totzubrennen". Diese wurde dann über Blaugel (r.F. ca. 25 %) aufbewahrt. Parallel zu den Versuchen der Fig. 3 wurden dann kleinere Mengen davon bei verschiedenen Feuchten klimatisiert. Danach wurde jeweils die Gewichtszu- oder Abnahme bestimmt (willkürlicher Bezugspunkt 20 % r.F.). In der gleichen Probe wurde anschliessend Wasser nach KARL FISCHER titriert. Ein Überschuss von Karl-Fischerlösung wurde nach einer Reaktionszeit von 30 Min. mit wasserhaltigem Methanol zurücktitriert. Es wurde mit der Apparatur zur Wasserbestimmung nach KARL FISCHER der Firma „Metrohm" gearbeitet.

7. Geschwindigkeit der Wasseraufnahme an der Luft (Tabelle III)

Drei 3.5 × 10 cm grosse Glasplatten wurden mit (a) 0.67; (b) 0.75; (c) 1.23 g Aluminiumoxid bestrichen. Das Verhältnis der Flächengewichte beträgt also 1:1.12:1.84.

* Firma Lambrecht, Göttingen.
** Firma DWM, Berlin, Modell LKL 25/70.

Nur bei (a) konnte die Schichtdicke zu 2 mm bestimmt werden; die anderen wurden mit dem Spatel aufgetragen.

8. Gewichtsverlust des Aluminiumoxids beim Erhitzen

Der Gewichtsverlust einer Probe Aluminiumoxid beim Erhitzen auf 800° wird thermogravimetrisch bestimmt. Eine wie unter E.6 vorbehandelte Probe wurde auf einer Thermowaage* bei einer Programmiergeschwindigkeit von 2.2°/Min. auf 800° erhitzt (vgl. Fig. 5).

DANK

Wir danken Frau COEN (Gruppe Metallurgie, Ispra) für die Messungen mit der Thermowaage. Wir danken auch Herrn Prof. E. STAHL, der uns den Beginn unserer Arbeiten in seinem Institut ermöglichte und den Fortgang der Untersuchungen durch wertvolle Ratschläge stimuliert hat.

Die Mehrzahl der genannten Polyphenyle wurden unter Euratom-Kontrakt Nr. 002-60-12-ETUB bei der Firma S.E.R.A.I. (Brüssel) synthetisiert[26].

ZUSAMMENFASSUNG

Für die Trennung von Polyphenylgemischen sind Aluminiumoxidschichten am geeignetsten. Die R_F-Werte und auch das effektive Trennergebnis hängen sehr stark von den klimatischen Bedingungen während der Trocknung und Entwicklung der Platten ab. Der Einfluss von relativer Feuchte und Temperatur wird mit zahlreichen Experimenten abgeklärt. Durch Vorklimatisierung der Schichten bei niederen Feuchten wird die Trennung der Terphenyle, bei hohen Feuchten die der „Hochsiedenden" begünstigt.

SUMMARY

For the separation of polyphenyls alumina layers are most efficient. The R_F values as well as the quality of the separations depend to a great extent on the hydrothermal conditions during drying and processing of the plates. The influence of the relative humidity and the temperature is elucidated by numerous experiments. Pre-conditioning at low relative humidities favours the separation of terphenyls, while preconditioning at higher humidities favours that of the "high boiling" polyphenyls.

LITERATUR

1 G. VOS, *Vortrag auf dem 7. Nuklearkongress, Rom* (Juni 1962).
2 R. T. KEEN, R. A. BAXTER, L. J. MILLER, R. C. SHEPARD UND M. A. ROTHERAM, *U.S. At. Energy Comm.*, NAA-SR-4356 (Jan. 1961).
3 R. T. KEEN, R. A. BAXTER, R. H. J. GERCKE UND W. L. ORR, *U.S. At. Energy Comm.*, NAA-SR-4355 (März, 1962).
4 R. BARRERA PINERO, *Junta Energia Nuclear*, 91-DQ/I 25, Madrid (1961).
5 T. M. SPOTSWOOD, *J. Chromatog.*, 2 (1959) 90.
6 W. W. WEST, *U.S. At. Energy Comm.*, AECU-4699 (1960).
7 H. HELLMAN, R. L. ALEXANDER UND C. F. COYLE, *Anal. Chem.*, 30 (1958) 1206.
8 L. SILVERMANN UND W. BRADSHAW, *Anal. Chem.*, 27 (1955) 96.
9 F. GEISS UND H. SCHLITT, *Euratom-Bericht*, EUR-19 d, (Nov. 1961).
10 F. GEISS UND S. SANDRONI, *Euratom-Bericht*, EUR-87 f (1962).
11 F. R. DUKE UND G. F. SMITH, *Ind. Eng. Chem., Anal. Ed.*, 12 (1940) 201.

* Firma D.A.M., Lyon, System Ugine-Eyraud. Modell B-60.

[12] *Anfärbereagenzien für Dünnschicht- und Papierchromatographie*, Merck-Bibliographie, S. 8.
[13] M. Brenner, A. Niederwieser, G. Pataki und A. R. Fahmy, *Experientia*, 18 (1962) 101.
[14] M. J. Normand und F. Geiss, *Vortrag auf dem 7. Nuklearkongress, Rom* (Juni 1962).
[15] H. Brockmann, *Angew. Chem.*, A 59 (1947) 201.
[16] E. B. Cornelius, T. H. Milliken, G. A. Mills und A. G. Oblad, *J. Phys. Chem.*, 59 (1955) 809.
[17] J. H. de Boer, J. J. Steggerda, J. M. H. Fortuin und P. Zwietering, *Proc. 2nd Intern. Congr. Surface Activity, London*, 1957, Vol. II, p. 93.
[18] *Fluka-Bulletin 1*, Alox-1-61-D; siehe auch H. Heinemann, K. A. Krieger und W. S. W. McCarter, *Ind. Eng. Chem.*, 38 (1946) 839.
[19] I. Jakubec, *Collection Czech. Chem. Commun.*, 24 (1959) 616.
[20] E. Stahl (Herausgeber), *Dünnschichtchromatographie*, Springer-Verlag, Berlin, 1962, Abb. 150.
[21] C. G. Scott, *J. Inst. Petrol.*, 45 (1959) 118.
[22] E. Stahl, *Pharm. Rundschau*, 1 (1959) 1.
[23] *Handbook of Chemistry and Physics*, 43. Aufl., Chemical Rubber Publ. Co., Cleveland, Ohio, S. 2536.
[24] D. S. Tarbell und T. Huang, *J. Org. Chem.*, 24 (1959) 887.
[25] Badger-McCabe-Kutzner, *Chemie-Ingenieurwesen*, Springer-Verlag, Berlin, S. 182.
[26] C. Beaudet, J. Horlait, A. Marcq und G. Belpaire, *Euratom-Bericht*, 31 f (1962).

LINEAR ELUTION ADSORPTION CHROMATOGRAPHY

VI. DEACTIVATED FLORISIL AS ADSORBENT

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INTRODUCTION

Preceding papers in this series have undertaken to develop a comprehensive and detailed theory of compound separation in linear elution adsorption chromatographic (LEAC) systems. Corresponding correlational equations have been derived from experimental retention volume data for the adsorbents alumina (Parts II–IV)[1–3] and silica (Part V)[4]. These equations permit the facile and systematic comparison of the separation possibilities in various LEAC systems[5,6], and suggest approximate generalizations for related non-linear chromatographic separations. The extension of this treatment to additional adsorbent types is of obvious practical importance, since it permits us to take advantage of the unique features of each adsorbent. As additional adsorbents are included in this semi-empirical study we should also expect to achieve greater insight into the dependence of adsorption separation on the fundamental characteristics of the adsorbent, and hence come to better understand the adsorption process itself.

The choice of Florisil (a commercial coprecipitate of silica and magnesia) as the subject of the present study was suggested by several considerations. Florisil has been widely used[7–11] in chromatographic separation, particularly in the analysis of petroleum and related substances[7–9]. Its pronounced acidic properties[12] (similar to those of silica/alumina cracking catalysts) distinguish it from the relatively neutral alumina and silica samples studied in Parts II–V[1–4], and suggest the possibility of unique separation capabilities. Finally, being composed largely of silica (82 % wt. %), it should be interesting to compare its adsorptive properties with those of silica.

EXPERIMENTAL

Samples of Florisil as received from the manufacturer (Floridin Company, Tallahassee, Fa.) were treated in the usual manner[1] prior to use as chromatographic adsorbent, *i.e.*, atmospheric calcination at 400° for 16 h, followed by equilibration with added liquid water for a minimum of 48 h. It has been observed[13] that uncalcined Florisil samples are contaminated by an adsorbed oil which must be washed from the adsorbent prior to its use in separation procedures. No such contaminant could be detected in our Florisil samples after calcination, using the strongest eluent of the present study (methylene chloride) as a wash material.

In contrast to our experience with alumina and silica, where the above procedure gives closely similar final adsorbent samples[1,4], two different shipments of Florisil showed markedly different adsorptive properties after calcination. This was traced to differences in the final surface area of calcined material, as summarized in Table I

TABLE I

SURFACE AREA AND PORE DIAMETER OF SOME FLORISIL SAMPLES

Sample	*Surface area* (m²/g)*	*Pore volume* (ml/g)*	*Average pore diameter* (Å)*
Calcined No. 1	155	—	—
Calcined No. 2	247	—	—
Manufacturer's values	298	0.461	62

* BET.

along with the manufacturer's values for original adsorbent. With the correction of retention volume data for these differing surface areas, as discussed in Part V[4] under "adsorbent standardization", the two adsorbents were then quite comparable. Whether these differences in final surface areas reflect corresponding differences in starting product or different sensitivities to the calcination procedure was not further investigated. This potential variability of the starting adsorbent restricts the usefulness of Florisil in *routine* analytical separation procedures[4,14].

CHEMISORPTION ON FLORISIL

Chemisorption in adsorption chromatographic systems, when it occurs, is an important problem, since the recoveries of solute from the column tend to be incomplete, and the elution bands show pronounced tailing. The chemisorption of basic nitrogen compounds on silica was noted in Part V[4], and attributed to the presence of acid groups on the adsorbent surface. The surface acidity of some commercial silicas has been related to the presence of free sulfuric acid[12] which can be removed by washing, so that chemisorption of basic compounds on silica may in principle be avoidable. The permanent acidity of Florisil[12] would be expected to lead to chemisorption of basic solutes, and ASATOOR AND DALGLIESCH[10] have noted that basic nitrogen compounds are strongly retained on Florisil. Reference also has been made to the semi-irreversible adsorption (chemisorption) of the hydrocarbon benzpyrene on Florisil[9]. Chemisorption on Florisil has since been confirmed in this laboratory, not only for basic nitrogen compounds such as the pyridines and quinolines, but also for such relatively non-basic substances as perylene and dimethyl terephthalate. The chemisorption of perylene on Florisil clearly involves an acid–base reaction, since adsorption bands develop the deep purple color associated with the dissolution of perylene in cold concentrated sulfuric acid. Chemisorption of dimethyl terephthalate on Florisil is illustrated in Table II and Fig. 1. Referring to Fig. 1, chemisorption of this solute in a chromatographic system using Florisil is shown to result in marked tailing of the elution band. At the same time, as seen in Table II, the recovery of solute from the column is incomplete and decreases markedly with

TABLE II

CHEMISORPTION OF DIMETHYL TEREPHTHALATE ON FLORISIL; SOLUTE RECOVERIES FROM FLORISIL AND SILICA

Solute/adsorbent (g/g)	R°		*Recovery (%)**	
	*Silica***	*Florisil****	*Silica***	*Florisil****
$2 \cdot 10^{-5}$	18.7	—	83	<5
$4 \cdot 10^{-5}$	18.0	14.4	84	18
$8 \cdot 10^{-5}$	18.2	16.0	91	15
$2 \cdot 10^{-4}$	—	14.2	—	38

* In first 60 ml/g of eluate.
** Elution by 40% methylene chloride–pentane from 16.0% H_2O–SiO_2 (Davison Code 12).
*** Elution by methylene chloride from 3.5% H_2O–Florisil (Sample No. 2).

decreasing sample size. Normal adsorption, by contrast, is shown (Table II, Fig. 2) for the elution of this same solute from a similar chromatographic system using silica rather than Florisil. The plot of Fig. 2 is typical of most LEAC systems, with the trailing edge of the elution band showing an exponential dependence of solute concentration on eluate volume. Table II shows the recovery of solute from the silica column as essentially complete, considering the probable purity of the solute, and independent of sample size. In view of the disadvantages of chemisorption in chromatographic separation, the apparently more frequent occurrence of chemisorption in separations using Florisil should be kept in mind when considering the use of this adsorbent.

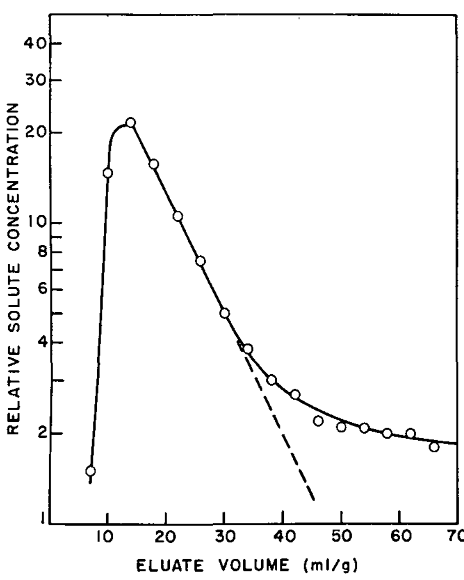

Fig. 1. Elution of dimethyl terephthalate from 3.5% H_2O–Florisil by methylene chloride; $2 \cdot 10^{-4}$ g/g column loading.

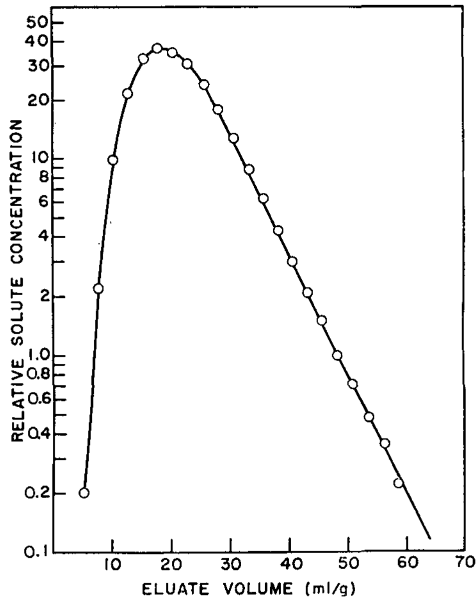

Fig. 2. Elution of dimethyl terephthalate from 16.0% H_2O–SiO_2 (Davison Code 12) by 40% v methylene chloride/pentane; $1.0 \cdot 10^{-4}$ g/g column loading.

The theoretical problem of calculating a retention volume from bands such as that of Fig. 1 has been discussed previously[4]. The elution band may be regarded as the superposition of a "physically adsorbed" band on top of a "chemisorbed band". The retention volume of the physically adsorbed band may be desired both from the theoretical standpoint for comparison with "normal" retention volumes obtained with non-chemisorbed solutes, and for establishing minimum values of the retention volume for chemisorbable species. The physical adsorption $\underline{R}^\circ$ value will of course always be less than the chemisorbed $\underline{R}^\circ$ value. In the latter connection, it should be noted that chemisorption of the *strongest* adsorbing component (or group of components) in a sample does not limit the analytical usefulness of a particular chromatographic system. Extrapolation of the trailing edge of the elution band as in Fig. 1 (dashed line) permits the calculation of the physical adsorption band and the corresponding $\underline{R}^\circ$ value. An essentially similar procedure was employed for the elution of aniline (chemisorbed) from silica[4]. This procedure has been shown to give $\underline{R}^\circ$ values equal to the "non-chemisorbed" K° values as required by theory. As seen in Table II, $\underline{R}^\circ$ values calculated in this fashion for Florisil are independent of sample size at low column loadings, as required by theory.

THE PREDICTION OF LEAC RETENTION VOLUME DATA

Correlational equations were presented in Parts IV[3] and V[4] for the prediction of retention volume values in chromatographic systems using alumina or silica as adsorbent. By combining these separate equations into one general form, it is found that the resulting unified equation describes the retention volume data for Florisil as well. It is convenient to break up the overall expression for predicting retention volume values into smaller equations as follows. First, the dependence of a linear equivalent retention volume $\underline{R}^\circ$ (ml/g) upon adsorbent surface volume V_a, adsorbent activity α, solute adsorption energy S°, eluent strength ε°, and solute area A_s is given as:

$$\log \underline{R}^\circ = \log V_a + \alpha (S^\circ - \varepsilon^\circ A_s) \tag{1}$$

The solute apparent area A_s is in turn given as the sum of solute group area contributions δ_i and group localization functions $c_1L_1\,(Q^\circ_i)$:

$$A_s = \sum^{i} [\delta_i + c_1 L_1\,(Q^\circ_i)] \tag{2}$$

Finally, the solute adsorption energy S° is related to solute group adsorption factors Q°_i, for various groups i, to geometry factors q°_j for various critical solute geometries j, to a localization function $c_2L_2\,(Q_k)$ characteristic of the single strongest adsorbing group k in the solute, to the number of atoms or groups n contained in aromatic ring systems, and to the number of such aromatic ring systems r not directly connected by aromatic carbon–carbon bonds (*e.g.*, $r = 2$ in the diphenyl alkanes):

$$S^\circ = \sum^{i} Q^\circ_i + \sum^{j} q^\circ_j - c_2 L_2\,(Q^\circ_k) \sum^{i \neq k} Q^\circ_i - c_3\,(n - 6r) \tag{3}$$

Certain of the above terms have been noted to be zero for alumina or for silica. The overall situation for alumina, silica, and Florisil is summarized in Table III, which

TABLE III

SUMMARY OF THE FORM OF THE TERMS OF EQUATIONS (1)–(3) WITH REFERENCE TO TABULATED VALUES FOR VARIOUS ADSORBENTS*

Term	*Alumina*	*Silica*	*Florisil*
V_a, α	II-2[a]	V-5[a]	VI-4[a], VI-(Fig. 3)
ε°	III-2, III-(Eqn. 2)	V-7, III-(Eqn. 2)	VI-9
$[\delta_i + c_1 L_1 (Q^\circ_i)]$	III-1[b]	V-12[c]	VI-9
δ_i [d]	III-1	III-1	III-1
$c_1 L_1 (Q^\circ_i)$ [d]	Zero	$14.6 f(Q^\circ_i)$[e]	$10 f(Q^\circ_i/1.6)$[e]
Q°_i	IV-1, IV-4, VI-11	V-6[f]	VI-11, VI-12[g]
q°_j	IV-3, VI-11, II-3[h] (refs. 15, 16)	VI-11	VI-11
$c_2 L_2 (Q^\circ_k)$	$f(Q^\circ_k)$[e]	$0.4 f(Q^\circ_k)$[e]	$0.65 f(Q^\circ_k)$[e]
c_3	Zero	0.11[i], 0.14[j]	Zero

* Roman numerals refer to the Part number in the present series (Part II = ref. 1; Part III = ref. 2; Part IV = ref. 3; Part V = ref. 4). Arabic numerals refer to the table number in the paper concerned.

[a] V_a calculable as 0.00035 (surface area in m^2/g) — 0.01 % H_2O.
[b] δ_i equal $[\delta_i + c_1 L_1 (Q^\circ_i)]$
[c] δ_i' equal $[\delta_i + c_1 L_1 (Q^\circ_i)]$
[d] To be used only if experimental value of $[\delta_i + c_1 L_1 (Q^\circ_i)]$ unavailable.
[e] $f(Q^\circ_k)$ tabulated in IV-2.
[f] and IV-1, where $(Q^\circ_i)_{sil} \cong (Q^\circ_i)_{alum}$.
[g] and IV-1, V-6, where $(Q^\circ_i)_{flor} \cong 1.6\ (Q^\circ_i)_{sil} \cong 1.6\ (Q^\circ_i)_{alum}$.
[h] q°_j values for aliphatic substituents on aromatic ring calculated on different basis than in VI-11.
[i] Small pore diameter gel (22 Å).
[j] Large pore diameter gel (170 Å).

notes the excludable terms in each case, and gives references for the location of the best tabulation of chromatograph parameters for each adsorbent (Roman numerals referring to paper number in present series, Arabic number referring to table number in that paper).

One change in Table III relative to Part V[4] should be noted. In Part V the coefficient c_2 for silica was postulated as zero. It has since been observed that a value of 0.4 for this coefficient significantly improves the accuracy of eqn. (3) in correlating experimental data (average deviation between experimental and calculated S° values for solutes of Table XVII, Part V, reduced from $\pm$ 0.33 to $\pm$ 0.24 log units). The significance of this change is discussed further in a later section.

As indicated in the experimental section, values of V_a cannot be tabulated as a function of water content for Florisil because of the variability of the surface area of starting calcined adsorbent. Consequently, the surface area of calcined adsorbent should be measured for each batch of adsorbent in order to calculate V_a. An analogous situation exists for silica samples, and Part V provides a detailed discussion of both the problem and its solution. For both alumina and silica, it has been possible to calculate the strength ε° of binary eluents from the ε° values of the constituent solvents. Application of this same equation to some binaries used as eluents with Florisil shows poorer reliability. Consequently, for accurate calculations it is necessary to experimentally determine the ε° values of binaries to be used with Florisil, following the procedure outlined in Part III[2].

As previously, a few examples of the calculation of retention volume using

Florisil as adsorbent will be given. In a first example, consider the elution of the hydrocarbon picene from a 1% H_2O–Florisil (whose initial calcined surface area was 155 m^2/g) by benzene. The value of V_a for this adsorbent may be calculated as 0.044 (*i.e.*, 0.00035 × 155 — 0.01). The value of α is given in Table IV of the present paper (or Fig. 3) as 0.63. The value of A_s can be calculated from eqn. (2) and Table IX of the present paper as 14 (6 + 0.5 × 16). The eluent parameter $\varepsilon°$ for benzene is given as 0.28 from Table IX. Substituting these values into eqn. (1):

$$\log \underline{R}° = \log 0.044 + 0.63\,(S° - 0.28 \times 14)$$

Now $S°$ must be calculated. $Q°_i$ for an aromatic carbon atom is 0.28 (Table XII), there are no $q°_j$ terms for the unsubstituted aromatic hydrocarbons, the strongest adsorbing group K is the aromatic carbon atom so $(Q°_i/1.6)$ is 0.17, and $f(0.17)$ from Table II of Part IV[3] is zero, and c_3 for Florisil is zero (Table III). Thus, from eqn. (3):

$$S° = (14 \times 0.28) + 0 - 0 - 0$$
$$= 6.16$$

Substituting the value 6.16 for $S°$ in the above expression for $\underline{R}°$, it is calculated that log $\underline{R}°$ equals 0.05. The experimental value was 0.16.

In a second example, we have the elution of methyl *m*-nitrobenzoate from a 3.5% H_2O–Florisil (initial calcined surface area equal 247 m^2/g) by methylene chloride. V_a is calculated as 0.051 (0.00035 × 247 — 0.035), and α is 0.51 (Table IV or Fig. 3). A_s is calculable from eqn. (2) and Table IX as 17.0 (6 × 1 + 6.0 + 5.0). $\varepsilon°$ for methylene chloride is 0.37, from Table IX. Substituting these values into eqn. (1):

$$\log \underline{R}° = \log 0.051 \pm 0.51\,(S° - 0.37 \times 17)$$

In the calculation of $S°$, there are 6 aromatic carbon atoms, 1 aromatic nitro group, and one aromatic ester group with respective $Q°_i$ values of 0.28, 4.88, and 5.47 (Table XII), no important geometry factors exist for this solute, the strongest adsorbing group K is the ester group so $f(5.47/1.6)$ is given as 0.41 from Table II in Part IV[3], and again c_3 is zero. Inserting these values into eqn. (3):

$$S° = (6 \times 0.28 + 4.88 + 5.47) + 0 - 0.65 \times 0.41 \times (1.68 + 4.88) - 0$$
$$= 10.28$$

Inserting this value of $S°$ into the previous expression for log $\underline{R}°$ gives a value of log $\underline{R}°$ equal 0.74. The experimental value was 0.94.

In a final example, consider the elution of indole from the same 1% H_2O–Florisil of example one by 15% methylene chloride–pentane. The V_a and α values have already been obtained, A_s is calculable as previously (6 × 1 + 2 × 0.5 + 10.4 = 17.4), and an experimental value of $\varepsilon°$ for this eluent and 1% H_2O–Florisil has been measured (0.123). Thus, from eqn. (1):

$$\log \underline{R}° = \log 0.044 + 0.63\,S° - (0.123 \times 17.4)$$

To calculate $S°$, the 8 aromatic carbon atoms have $Q°_i$ values of 0.28 and the nitrogen

group has a Q°_{i} value of 4.84, there are no q°_{j} terms, and the strongest adsorbing group K is the –NH– group. $f(4.84/1.6)$ is 0.34, and c_3 is zero, so:

$$S^{\circ} = (8 \times 0.28 + 4.84) + 0 - 0.65 \times 0.34 \times 0.56 - 0$$
$$= 6.96$$

Substituting into the above expression the calculated value of log $\underline{R}^{\circ}$ is 0.89. The experimental value was 0.96.

If an experimental value of $\alpha\varepsilon^{\circ}$ had been unavailable, the ε° values of pentane (0.00) and methylene chloride (0.37) could have been used to estimate an $\alpha\varepsilon^{\circ}$ value according to eqn. (2) of Part III[2]. This calculated $\alpha\varepsilon^{\circ}$ value (0.099) is sufficiently in error to give a distinctly poorer final value of log $\underline{R}^{\circ}$ (1.24).

THE ROLE OF ADSORBENT ACTIVITY AND SURFACE VOLUME

The effect of adsorbent activity α and surface volume V_a on retention volume is given in eqn. (1), which has been previously verified for alumina[1] and silica[4]. Assuming the applicability of eqn. (1) to Florisil as adsorbent, values of V_a can be calculated from BET surface area data and the amount of water added to calcined adsorbent as in Part V[4] (eqn. 3). Values of α for given adsorbent samples can in turn be calculated from $\underline{R}^{\circ}$ values for a standard solute and eluent (naphthalene–pentane in previous work) if the value of α for calcined adsorbent is defined as 1.00. Fig. 3 and Table IV

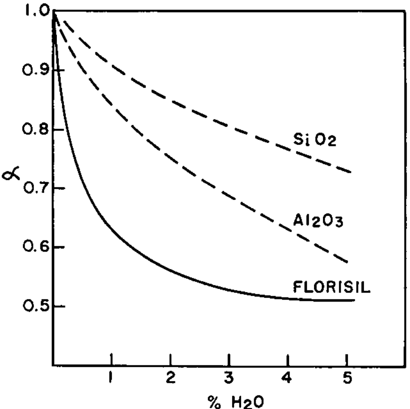

Fig. 3. Adsorbent activity functions α for Florisil, alumina, and silica *versus* percent added water.

summarize values of α for the two Florisil samples studied in the present investigation, using both naphthalene and fluoranthene as standard solutes, and pentane as standard eluent. The abrupt initial decline in α with added water is believed to result from the Florisil strong acid sites, which are preferentially covered by the first 1 % or so of added water. Fig. 3 contrasts the behavior of Florisil in this regard with the adsorbents alumina and silica (dashed lines).

Table V summarizes data on a number of chromatographic systems designed to test the applicability of eqn. (1) to Florisil as adsorbent. Values of V_a and α were

TABLE IV

ADSORBENT PARAMETERS FOR PRESENT TWO FLORISIL SAMPLES

Sample		α	V_a
No. 1	Calcined	1.00	0.054
	1 % H_2O	0.63	0.044
	5 % H_2O	0.52	0.013*
No. 2	Calcined	1.00	0.086
	0.5 % H_2O	0.73	0.081
	1.0 % H_2O	0.62	0.076
	3.5 % H_2O	0.51	0.051
	7.0 % H_2O	0.50	0.022*

* Chromatographically evaluated[1,4] since surface coverage by water is near complete[4].

TABLE V

TEST OF EQUATION (1) FOR FLORISIL AS ADSORBENT; FLORISIL SAMPLE NO. 2 USED

Chromatographic system	*log R°*					$(S^\circ - \varepsilon^\circ A_S)$
	0 % H_2O	*0.5 % H_2O*	*1.0 % H_2O*	*3.5 % H_2O*	*7.0 % H_2O*	
Benzene–pentane						
(Exptl.)	0.62	0.27	—0.05	—0.40		1.75
(Calc.)	0.68	0.19	—0.04	—0.40		
Naphthalene–pentane						
(Exptl.)	1.77	1.00	0.64	0.17	0.16	2.86
(Calc.)	1.79	1.00	0.65	0.17	0.23	
Fluoranthene–pentane						
(Exptl.)		2.19	1.69	1.06	0.60	4.60
(Calc.)		2.29	1.73	1.06	0.64	
Perylene–pentane						
(Exptl.)			2.26	1.65	1.20	5.76
(Calc.)			2.45	1.65	1.22	
p-Xylene–pentane						
(Exptl.)	1.63	0.91	0.17	0.30		1.94
(Calc.)	0.87	0.33	0.08	0.30		
Phenyl ethyl sulfide–pentane						
(Exptl.)		1.52	1.26	0.76	0.39	4.02
(Calc.)		1.84	1.37	0.76	0.35	
Picene–benzene						
(Exptl.)	0.45	0.41	0.26	—0.01	—0.24	2.51
(Calc.)	1.44	0.74	0.44	—0.01	—0.41	
1-Nitronaphthalene–benzene						
(Exptl.)			1.16	0.40	—0.02	3.31
(Calc.)			0.93	0.40	—0.01	
o-Nitroaniline–benzene						
(Exptl.)		1.96	1.80	1.15	0.73	4.78
(Calc.)		2.40	1.84	1.15	0.73	
Acetophenone–methylene chloride						
(Exptl.)			1.20	0.42	0.06	3.35
(Calc.)			0.96	0.42	0.12	
α*	1.00	0.73	0.62	0.51	0.50	
V_a*	0.086	0.081	0.076	0.051	0.022	

* Data of Table IV.

evaluated as above. Values of ($S° - \varepsilon° A_s$) for each system were calculated from the $\underline{R}°$ value for elution from 3.5 % H_2O–Florisil, and values of $\underline{R}°$ for other adsorbent activities derived from this value of ($S° - \varepsilon° A_s$) and eqn. (1). It is apparent that the calculated and derived $\underline{R}°$ values are not in good agreement. The overall average deviation is $\pm$ 0.13 log units, and several values disagree by more than 0.5 log units. If the data are divided into two sets, one including data for calcined and 0.5 % H_2O–Florisil, and the other set including the remaining data for Florisil deactivated by one or more percent water, the data for the less deactivated adsorbent samples show an average deviation between experimental and calculated $\underline{R}°$ values equal to $\pm$ 0.33 log units, while the more highly deactivated adsorbents show a corresponding average deviation of only $\pm$ 0.06 log units. It may be concluded that eqn. (1) is unreliable for Florisil samples containing less than 1 % added water, but that data for other adsorbent activities are correlated with reasonable accuracy. The reason for the failure of eqn. (1) in the present connection is not difficult to discover. The fundamental basis of eqn. (1) has been discussed elsewhere[1,16]. Briefly, eqn. (1) assumes adsorption sites of continuously varying energy and basically similar character. That is, eqn. (1) would not be expected to apply when the strong sites on an adsorbent surface are of fundamentally different character (*e.g.*, involving bonding of varying type, as electrostatic polarization in one case, charge transfer in another, or covalent bonding in a third case). Evidence has already been presented, however, to indicate that precisely this situation characterizes the Florisil surface; that is, coverage by strong (acid) sites which are fundamentally different from the "normal" sites (which presumably form simple electrostatic polarization bonds with the solute[16]). Fig. 3 suggests that the strong acid sites are largely covered up after the addition of 1 % water to calcined Florisil, with the remaining surface being more nearly "normal" by comparison with the surfaces of alumina and silica. The applicability of eqn. (1) to moderately deactivated Florisil samples similarly suggests that the removal of acid sites by the first 1 % added water leaves a "normal" adsorbent surface.

The failure of eqn. (1) to apply to calcined and lightly deactivated Florisil does not necessarily limit the usefulness of calcined adsorbent. The separation possibilities with calcined Florisil are in fact actually expanded over the case where eqn. (1) is applicable to an adsorbent. Thus, in "normal" chromatographic systems such as those using alumina, silica, and moderately deactivated Florisil, separation order is not a function of adsorbent activity, and in general separation will be unimproved by changes in activity so long as the necessary cut points occur at reasonably large eluate volumes (greater than 1 ml/g). Failure to obtain a desired separation using deactivated Florisil, however, may well be overcome on changing to calcined or near calcined adsorbent. Unfortunately, the data of Table V offer no obvious clues as to when such a change will be advantageous.

THE ROLE OF THE ELUENT

Retention volume data are presented in Tables VI and VII for the elution of a number of hydrocarbons from Florisil samples of varying activity and from both of the batches described in Table I, by several eluents. The assumption that A_S is given as $6 + {}^1/_2\,(n - 6)$, as for elution from alumina and silica, gives an excellent fit of these data to eqn. (1), $\pm$ 0.05 log units, when the indicated values of $S°$ for each solute and

TABLE VI

EFFECT OF ELUENT ON ELUTION OF AROMATIC HYDROCARBONS FROM FLORISIL (SAMPLE NO. 1)

Solute		log $\underline{R}°$									$S°$
	Water on adsorbent α* V_a*	0.0% 0.99 0.054	1.0% 0.63 0.044							5% 0.52 0.013	
	Eluent**	P	P	5% M-P	15% M-P	50% M-P	10% B-P	25% B-P	B	P	
	$\alpha\varepsilon°$	0.000	0.000	0.066	0.123	0.195	0.094	0.132	0.177	0.000	
Benzene	(Exptl.)	0.17	—0.11								1.71
	(Calc.)	0.44	—0.28								
Naphthalene	(Exptl.)	1.59	0.46							—0.51	2.88
	(Calc.)	1.61	0.45							—0.39	
Acenaphthylene	(Exptl.)		0.78	0.19							3.39
	(Calc.)		0.78	0.19							
Phenanthrene	(Exptl.)		1.11	0.45			0.10			0.13	3.88
	(Calc.)		1.08	0.42			0.17			0.13	
Fluoranthene	(Exptl.)		1.40	0.70	0.04		0.34				4.47
	(Calc.)		1.45	0.72	0.10		0.43				
1,2-Benzanthracene	(Exptl.)		1.78	0.94	0.22		0.46	0.06		0.63	4.83
	(Calc.)		1.68	0.89	0.20		0.55	0.10		0.63	
Perylene	(Exptl.)			1.18	0.51		0.87	0.26			5.40
	(Calc.)			1.18	0.44		0.82	0.32			
Picene	(Exptl.)			1.69	0.89	—0.02	1.53	1.07	0.16		6.46
	(Calc.)			1.79	0.99	—0.02	1.39	0.86	0.23		

* Data of Table IV.
** Symbols defined as follows: P, pentane; B, benzene; M, methylene chloride.

TABLE VII

EFFECT OF ELUENT ON ELUTION OF AROMATIC HYDROCARBONS FROM FLORISIL (SAMPLE NO. 2)

Solute		log $\underline{R}°$				$S°$
	Water on adsorbent α* V_a*	3.5% 0.51 0.051				
	Eluent** $\alpha\varepsilon°$	P 0.000	10% M-P 0.059	20% M-P 0.097	30% M-P 0.127	
Naphthalene	(Exptl.)	0.17				2.88
	(Calc.)	0.18				
Phenanthrene	(Exptl.)	0.68				3.88
	(Calc.)	0.69				
1,2-Benzanthracene	(Exptl.)	1.25	0.46	0.01	—0.41	4.83
	(Calc.)	1.17	0.46	0.01	—0.35	
Picene	(Exptl.)		1.18	0.67	0.29	6.46
	(Calc.)		1.18	0.64	0.22	

* Data of Table IV.
** Symbols defined as follows: P, pentane; M, methylene chloride.

TABLE VIII

EFFECT OF ELUENT ON ELUTION OF NON-HYDROCARBONS FROM FLORISIL

Solute	*Water on adsorbent Sample* a^* V_a^*		*log* $\underline{R}°$													$S°$
			1.0% No. 1 0.63 0.044							*5.0% No. 1 0.52 0.013*	*3.5% No. 2 0.51 0.051*					
	*Eluent***		*P*	*5% M*	*15% M*	*50% M*	*10% B*	*B*	*M*	*B*	*10% M*	*20% M*	*30% M*	*B*	*M*	
	$a\varepsilon°$		*0.000*	*0.066*	*0.123*	*0.195*	*0.094*	*0.177*	*0.233*	*0.146*	*0.059*	*0.097*	*0.127*	*0.143*	*0.188*	
Phenyl ethyl sulfide	(Exptl.)	8.5	1.06	0.41	0.05		0.15									3.78
	(Calc.)	(8.5)	1.02	0.46	—0.02		0.22									
Phenetole	(Exptl.)	10.3	1.49	0.82	0.41		0.57	—0.50			0.43	0.06	—0.17			4.59
	(Calc.)	(10.3)	1.53	0.85	0.26		0.56	—0.27			0.44	0.05	—0.26			
Nitrobenzene	(Exptl.)	12.0			1.52	0.74		0.76	0.16	—0.41	1.28	0.87	0.59	0.19	—0.43	6.59
	(Calc.)	(12.0)			1.31	0.45		0.67	—0.01	—0.21	1.36	0.91	0.55	0.35	—0.18	
Methyl benzoate	(Exptl.)	11.0				1.23		1.30	0.72	0.12	1.68		1.01	0.73	0.12	7.18
	(Calc.)	11.0				1.01		1.22	0.60	0.23	1.72		0.97	0.80	0.30	
Acetophenone	(Exptl.)	14.2								0.17	2.16	1.77	1.46	1.06	0.42	8.57
	(Calc.)	14.2								0.50	2.24	1.70	1.28	1.05	0.41	
p-Diethoxybenzene	(Exptl.)	14.2				0.65				—0.23		1.12	0.85			7.58
	(Calc.)	14.6				0.57				—0.08		1.16	0.73			
m-Dinitrobenzene	(Exptl.)	17.4							0.85			1.96	1.65		0.34	10.02
	(Calc.)	18.0							0.76			2.07	1.53		0.44	
Indole	(Exptl.)	17.7			0.96		1.34	—0.08								6.96
	(Calc.)	17.7			0.89		1.40	—0.05								

* Data of Table IV.

** Symbols defined as follows: P, pentane; B, benzene; M, methylene chloride; % M, % B refer to M/P and B/P binaries; % refers to volume percent

for each eluent are assumed. The behavior of the eluent in the elution of hydrocarbons from all three of the adsorbents so far studied is therefore essentially identical, and eqn. (1) applies without comment.

The evaluation of $\alpha\varepsilon^\circ$ for the eluent–adsorbent combinations of Tables VI and VII from these hydrocarbon $\underline{R}^\circ$ data permits in turn the evaluation of A_s values for the non-hydrocarbons, as was done in the case of silica (Part V)[4]. In Table VIII, data for the elution of several non-hydrocarbons from various Florisil samples by several eluents are summarized. The $\alpha\varepsilon^\circ$ values of the eluent systems described in Tables VI and VII are taken from those tables. The other values of $\alpha\varepsilon^\circ$ are the averages of extrapolating log $\underline{R}^\circ$ values *versus* $\alpha\varepsilon^\circ$ from known to unknown eluents. Finally, the experimental values of A_s shown are the best (least squares) fit to all of the data for a given solute.

The best experimental values of A_s for the monosubstituted benzenes were used to calculate values of $[\delta_i + c_1L_1\,(Q^\circ{}_i)]$ for each substituent group (equal $A_s - 6$), and the values of A_s for the polysubstituted benzenes then calculated as the sum of group contributions. The overall agreement between experimental and calculated $\underline{R}^\circ$ values, using these resulting A_s values, was $\pm$ 0.11 log units, which is reasonable agreement. As in the case of elution from silica, it is apparent that the non-hydrocarbon A_s values are considerably larger than would have been expected from their actual areas ($\sum\limits^{i} \delta_i$), and that the $c_1L_1\,(Q^\circ{}_i)$ term of eqn. (2) is non-zero for Florisil. In this respect, Florisil resembles silica rather than alumina. Table IX summarizes

TABLE IX

SOLUTE GROUP APPARENT AREAS ON FLORISIL

Group	$[\delta_i + c_1L_1(Q^\circ{}_i)]$	
	Exptl.	*Calc.**
–CH=	0.5–1.0**	0.5–1.0**
–SR	2.5	2.7
–OR	4.3	3.3
$-NO_2$	6.0	6.1
$-CO_2R$	5.0	7.1
–COR	8.2	6.5
–NH–	10.4	(4.0)

* $\delta_i + 10 f(Q^\circ{}_i/1.6)$.
** See ref. 2.

Eluent parameter values

Solvent	ε°		
	Florisil	*Alumina*	*Silica*
Pentane	0.00	0.00	0.00
Carbon tetrachloride*	0.07	0.18	0.14
Benzene	0.28	0.32	0.25
Chloroform*	0.31	0.40	—
Methylene chloride	0.37	0.42	0.32
Di-ethyl ether*	0.49	0.46	—

* Florisil values based on limited unreported data.

experimental values of $[\delta_i + c_1 L_1 (Q^\circ_i)]$ for the various solute groups of Tables VI–VIII. With the exception of the –NH– group, which differs from the other strong groups in forming part of an aromatic ring, these apparent group area values are well represented by the relationship:

$$\text{Group area} = \delta_i + 10 f (Q^\circ_i/1.6) \quad (4)$$

$f(x)$ refers to the localization function previously defined for alumina in Part IV[3]. The form of this latter expression, specifically the factor 1/1.6, will be rationalized in the following section.

Eluent strength values ε° for the three solvents presently studied, as well as values from limited unreported data on other solvents, are also summarized in Table IX. Comparison with corresponding ε° values for alumina and silica shows reasonable agreement.

THE ROLE OF SOLUTE STRUCTURE

The preceding discussion and data provide a basis for evaluating experimental S° values and hence arriving at the dependency of solute $\underline{R}^\circ$ values on solute structure, as in Part V[4]. The relationship between $\underline{R}^\circ$ values and molecular structure for unsubstituted aromatic hydrocarbons has been shown to be of one form (linear) with alumina[1,15] and another (non-linear) with silica[4,17]. This behavior has been related to the way strong sites are distributed on the adsorbent surface[17]. In the case of alumina, it is believed that the strongest sites are randomly distributed, while for silica it appears that the strongest sites occur in clumps roughly the size (40 Å^2) of a benzene molecule. This grouping of sites on silica gives rise to the $c_2 (n - 6r)$ term of eqn. (3). S° values for the elution of various unsubstituted aromatic hydrocarbons from Florisil have been derived from the data of Tables VI and VII, and tabulated therein. These S° values are plotted *versus* carbon number n in Figure 4. It is evident

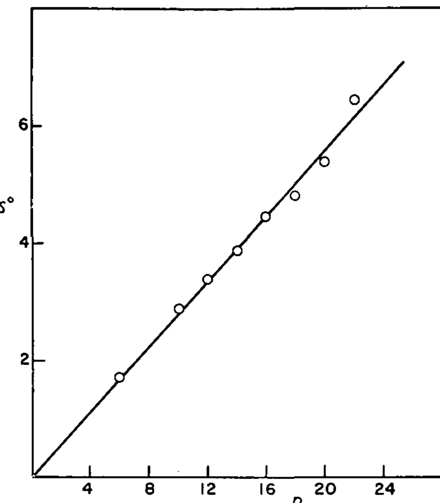

Fig. 4. Dependence of S° on carbon number for unsubstituted aromatic hydrocarbons; Florilsi adsorbent.

that these Florisil $S°$ values are linear in n for $n \leqslant 22$, and Florisil resembles alumina rather than silica in this respect. Consequently, the term c_2 $(n - 6r)$ may be neglected in the correlation and prediction of Florisil $\underline{R}°$ values.

The effect of alkyl substitution on the $\underline{R}°$ values of the aromatic hydrocarbons eluted from Florisil is summarized in Tables X and XI. The experimental $\underline{R}°$ data

TABLE X

RETENTION VOLUMES OF THE ALKYL-SUBSTITUTED BENZENES; PENTANE ELUTION FROM 1.0% H_2O–FLORISIL (SAMPLE NO. 2)

Solute	*log $\underline{R}°$*	
	Exptl.	*Calc.**
Benzene	−0.05	−0.05
Toluene	+0.03	+0.05
Ethylbenzene	+0.02	+0.04
n-Butylbenzene	+0.04	+0.05
n-Dodecylbenzene	+0.10	+0.10
o-Xylene	+0.26	+0.25
p-Xylene	+0.17	+0.15
o-Diethylbenzene	+0.26	+0.23
p-Diethylbenzene	+0.17	+0.13
Pentamethylbenzene	+0.94	+0.96
Hexamethylbenzene	+1.17	+1.16
Tetralin	+0.42	+0.42

* Using parameters of Table XI.

of Table X for various alkyl-substituted benzenes were used to derive the Florisil $Q°_i$ and $q°_j$ values of Table XI for alkyl substituent groups and geometries. These values were then used in the calculation of the $\underline{R}°$ data of Table X, assuming the value for benzene. The average deviation between experimental and calculated values of log $\underline{R}°$ is only $\pm$ 0.02 units. The Florisil alkyl substitution parameters of Table XI are also compared with corresponding alumina and silica values from Parts II[1] and V[4]. The $Q°_i$ values for Florisil are in the same order as for alumina, but smaller. The various Florisil geometry effects ($q°_j$ values) parallel those for alumina and silica, and tend to be 2–3 times larger.

Table XII summarizes the Florisil $Q°_i$ values for the various solute groups so

TABLE XI

HYDROCARBON SOLUTE PARAMETERS FOR FLORISIL, ALUMINA, AND SILICA

Parameter	*Florisil*	*Alumina*	*Silica*
$Q°_i$, $-CH_2-$ (alkyl)	+0.013	+0.02	−0.05
$Q°_i$, $-CH_2-$ (aromatic)	+0.16	+0.07	+0.01
$Q°_i$, $-CH_3$ (alkyl)	−0.02	−0.03	+0.05
$Q°_i$, $-CH_3$ (aromatic)	+0.16	+0.06	+0.11
Ortho alkyl substitution	+0.16	+0.09	+0.09
Cyclo alkyl ring closure	+0.24	+0.08	+0.14

TABLE XII

SOLUTE GROUP ADSORPTION FACTORS (Q°_{i}) ON FLORISIL

Group	Q°_{i}			
	Florisil		Silica[4]	1 % H_2O-Florisil
	Exptl.	Calc.*		
–CH=	0.28	0.4	0.25	0.18
Ar)–SR	2.07	2.1	1.29	1.30
Ar)–OR	2.88	2.9	1.83	1.81
Ar)–NO_2	4.88	4.4	2.77	3.07
Ar)–CO_2R	5.47	5.6	3.45	3.45
Ar)–COR	6.86	7.5	4.69	4.32
Ar)–NH–(Ar	4.84	4.8	3.00	3.05
R)–CH_2–(R	0.013			

* 1.6 $(Q^{\circ}_{i})_{sil}$.

far studied. These are definitely larger than the corresponding alumina or silica values. The relationship:

$$(Q^{\circ}_{i})_{flor} = 1.6\ (Q^{\circ}_{i})_{sil}$$

gives a good fit of the Florisil values (average deviation, $\pm$ 0.2 units), with only the aromatic carbon group showing a relatively poor calculated value. This suggests that the surface energy of calcined silica is comparable to a Florisil surface with α equal to 1/1.6 or 0.63. This corresponds to a 1 % H_2O–Florisil sample, and it is tempting to relate this observation to our preceding remarks on the coverage of strong acid sites by the first 1 % of added water in the case of starting calcined Florisil. Thus, the calcined Florisil surface may be regarded (in one sense) as a calcined silica surface on which are superimposed strong acid sites. This is perhaps not unreasonable when it is recalled that Florisil is largely silica. The strong sites are for the most part covered by

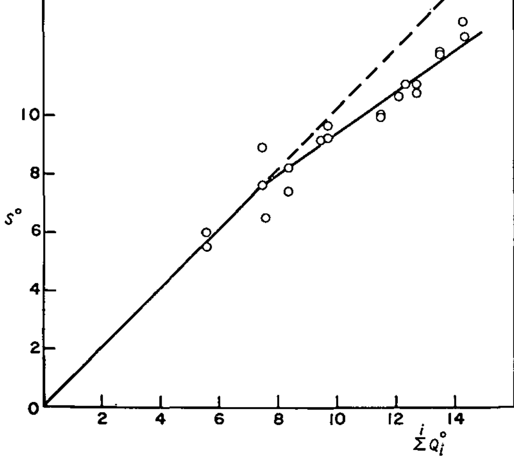

Fig. 5. Localization as a factor in determining the S° values of strongly adsorbed solutes; Florisil adsorbent.

the first 1 % added water, leaving essentially a calcined silica surface. This view of the Florisil surface must of course represent only a crude approximation.

Table XIII summarizes experimental Florisil $S°$ values for a range of solutes, $\underline{R}°$ for most of which have been given in preceding tables. In Fig. 5 these $S°$ values are plotted *versus* the sum of solute group adsorption factors $\overset{i}{\Sigma}\, Q°_i$. This provides a test for localization of the solute, as in the case of alumina[3]. As seen in Fig. 5, the experimental plot breaks rather definitely at $S° \approx 8$, indicating the necessity of including the $c_2L_2\,(Q°_k) \overset{i \neq k}{\Sigma}\, Q°_i$ term of eqn. (3) in calculations for Florisil.

Fig. 5, which should be compared to Fig. 9 and 10 of Part V[4] (and related discussion), confirms the importance of localization and its effect on $S°$ for Florisil as adsorbent. The $c_2L_2\,(Q°_k)$ term for Florisil is therefore non-zero. This Florisil term is well approximated by the relationship:

$$c_2L_2\,(Q°_k) = 0.65\, f\,(Q°_i/1.6) \tag{5}$$

where $f(x)$ is the same functional relationship defined for alumina[3]. The differing form of the above relationship ($c_2 \neq 1$, $1/1.6$ factor), which should be compared with eqn. (4), is logical if we recall our above remarks on the larger $Q°_i$ values for Florisil relative to silica. Thus, the localization function should be related to the energy of adsorption sites, and should assume similar values when adsorbent surfaces of similar energies (*e.g.*, silica and alumina) are involved. Since 1 % H_2O–Florisil is comparable in this respect to calcined alumina and silica, the localization function $f(x)$ should be defined relative to $Q°_i$ values defined for this Florisil activity (set α equal 1.00 for 1.0 % H_2O–Florisil). This is equivalent to dividing the actual $Q°_i$ values (defined relative to calcined Florisil) by 1.6 as in eqn. (4) and (5). The c_2 value of 0.65 for 1.0 % H_2O–Florisil appears consistent with the resemblance of Florisil to both alumina ($c_2 = 1.00$) and silica ($c_2 = 0.40$) in other respects, and hence is intermediate between the other two adsorbents.

The adequacy of the localization term of eqn. (5) is tested in Table XIII by comparing experimental and calculated $S°$ values. The latter are simply $[\overset{i}{\Sigma}\, Q°_i - f\,(Q°_i/1.6) \overset{i \neq k}{\Sigma}\, Q°_i]$. The average deviation is $\pm$ 0.4 units, which is reasonable when it is noted that the $q°_j$ terms involved in isomer $S°$ values have been ignored, and the experimental variation in isomer $S°$ values is $\pm$ 0.3 units.

COMPARISON OF FLORISIL, ALUMINA, AND SILICA AS CHROMATOGRAPHIC ADSORBENTS

With the completion of the present experimental study, it is appropriate to compare the separation capabilities of the three adsorbents so far investigated. This is conveniently done by reviewing the dependence upon adsorbent type of each of the terms in eqns. (1–3). The adsorbent parameters V_a and α measure the effect of water content on the separation characteristics of the adsorbent. Since the separation sequence of a group of solutes is determined solely by the $(S° - \varepsilon° A_s)$ term of eqn. (1), it is apparent that changes in V_a or in α cannot be relied on to separate solute pairs having the same $\underline{R}°$ values at one adsorbent activity. The ex-

TABLE XIII

SOLUTE S° FACTORS FOR ELUTION FROM FLORISIL

Solute	*System**	*log $\underline{R}^\circ$*	S°		
			Axptl.	*Calc.***	$\sum^{i} Q^\circ_i$
p-Diethoxybenzene	A	—	7.6	7.2	7.4
o-Diethoxybenzene	B	1.39	8.9	7.2	7.4
1-Methoxynaphthalene	C	0.34	5.5	5.6	5.7
2-Methoxynaphthalene	C	0.69	6.0	5.6	5.7
m-Dinitrobenzene	A	—	10.0	10.3	11.4
p-Dinitrobenzene	D	0.38	9.9	10.3	11.4
p-Nitroanisole	D	0.20	9.1	8.8	9.4
2,4-Dinitroanisole	D	1.00	12.6	12.6	14.4
2,4-Dimethoxynitrobenzene	D	0.58	11.0	11.0	12.3
1-Nitronaphthalene	C	0.67	6.5	7.4	7.7
Dimethyl terephthalate	D	1.17	10.7	11.2	12.6
Dimethyl phthalate	D	1.32	11.0	11.2	12.6
Methyl *m*-nitrobenzoate	D	0.94	10.7	10.8	12.0
Methyl 1-naphthoate	E	0.60	7.4	8.0	8.4
Methyl 2-naphthoate	E	1.03	8.2	8.0	8.4
1-Acetonaphthone	E	1.15	9.3	9.3	9.7
2-Acetonaphthone	E	1.45	9.9	9.3	9.7
2,5-Dimethoxyacetophenone	D	1.26	13.1	12.6	14.3
m-Nitroacetophenone	D	1.06	12.1	12.0	13.4
p-Nitroacetophenone	D	1.05	12.0	12.0	13.4

* A: See Table VIII; B: 30% v methylene chloride–pentane; 3.5% H_2O–Florisil (No. 2); C: 20% v methylene chloride–pentane; 3.5% H_2O–Florisil (No. 2); D: Methylene chloride; 3.5% H_2O–Florisil (No. 2); E: Benzene; 3.5% H_2O–Florisil (No. 2).

** $\sum^{i} Q^\circ_i - 0.65 f(Q^\circ_k/1.6) \sum^{i \neq k} Q^\circ_i$.

ception in the case of 0–1% H_2O–Florisil has already been noted. Assuming that the $(S^\circ - \varepsilon^\circ A_s)$ terms for two solutes differ, however, both V_a and α can play a role in the relative separability of the solute pair. In general, the larger is α the larger will be the retention volume ratio for the two solutes, and the better the separation. This consideration must be weighed against possible reduced linear capacity in more active adsorbents[5,18], and inconveniently large cut point volumes in the separation. Both alumina and silica have comparable ranges in α (1.0–0.6) achievable by varying water content, but the larger linear capacity of calcined large surface area silica samples[5] somewhat increases their *practical* range in α values. Florisil, on the other hand, because of the necessity of deactivating with one or more percent of added water, has a very small range (0.63–0.50) in possible α values.

Differences in adsorbent V_a values play a more important role in determining the potential separation of certain mixtures, notably those of weakly adsorbing solutes. In this case, the various $(S^\circ - \varepsilon^\circ A_s)$ values may be sufficiently small, although differing appreciably in value, to make the resulting $\underline{R}^\circ$ values all less than or equal unity, even for the largest α values. In general, solute mixtures where $\underline{R} \leqslant 1$ for all solutes cannot readily be separated. If the $(S^\circ - \varepsilon^\circ A_s)$ terms for each solute in such a mixture are comparable between adsorbents, and if the maximum α values possible for each adsorbent are similar, then the V_a terms will control the "goodness"

of separation, the larger V_a value giving the better separation (or the only separation). An example is provided by the separation of aliphatic mono-olefins from saturated hydrocarbons. Only large surface area adsorbents (with correspondingly large V_a values) are capable of this separation[5]. Among the three commercial adsorbent types so far studied, the maximum V_a values are: Davison Code 12 silica (calcined), 0.30; Florisil (1 % water), 0.08; Alcoa F-20 alumina (calcined), 0.06. The code 12 silica is obviously the best of these adsorbents for the separation of very weakly adsorbing mixtures.

The separation of two or more solutes or solute types ultimately requires significant differences in either their $S°$ (adsorption energy) or A_s (effective area) values. In comparing the three adsorbent types with respect to selected separations, it is useful to classify the compound types so far studied as follows: (I) unsubstituted and alkyl-substituted aromatic hydrocarbons; (II) other substituted aromatic hydrocarbons; (III) substituted aliphatics; (IV) pyrrole derivatives; (V) basic nitrogen compounds. Separations of these various compound types, both within and between various classes, may be further subdivided according to whether separation by compound type (groups of similar compounds) or separation between individual compounds is desired.

Separation of the unsubstituted and alkyl-substituted aromatic hydrocarbons (I) by type (*e.g.*, alkylbenzenes, alkylnaphthalenes) is best done using alumina, primarily because the alkyl substitution parameters ($Q°_i$, $q°_j$) for alumina are so much smaller than for silica or Florisil (see Table XI). Additionally, the c_3 $(n - 6r)$ term for silica (eqn. 3) leads to decreased differences between the $S°$ values of the unsubstituted aromatic hydrocarbons, with further worsening of the separation of aromatic types using silica. Conversely, the separation of individual alkyl aromatics should be best accomplished with Florisil, because the alkyl substitution parameters (Table XI) are by far the largest. The separation of individual unsubstituted aromatic hydrocarbons, and particularly isomers, has not been extensively investigated, except in the case of alumina[15,16]. It appears, however, that alumina *is* the preferred adsorbent in this capacity, and many successful separations of this type by alumina have been reported.

Aromatic hydrocarbons substituted by groups other than alkyl (II) constitute the most extensively studied group of solutes in the present series of papers. Table XIV has been prepared to focus attention on the many differences in separation order which arise among the three adsorbent types. Values of the solute parameters $S°$ and A_s were calculated for each of the solutes of Table XIV, and these were used to calculate the term $(S° - \varepsilon° A_s)$ for eluent strengths ($\varepsilon°$) of 0.00 and 0.30. Differences in the $(S° - \varepsilon° A_s)$ term between two solutes in the same chromatographic system (same adsorbent, eluent) are a good index of the separability of that solute pair. The term $(S° - \varepsilon° A_s)$ for Florisil was multiplied by the α value for 1 % H_2O–Florisil (0.63), since this is the highest adsorbent activity for which these data are applicable.

As previously pointed out, the solute group $Q°_i$ values for the three adsorbents are all quite comparable, at least when the values of $Q°_i$ for Florisil are expressed, as in Table XIV, on a 1 % water–adsorbent basis. Consequently, the major differences in separation order of a series of solutes of the type C_6H_5–X, C_6H_4–X_2, C_6H_3–X_3 are determined by the localization of strong solute groups, *for elution by pentane or other weak eluents*. In such cases, silica is better than Florisil which is better than alumina.

TABLE XIV

SOLUTE/ELUENT PARAMETERS FOR SUBSTITUTED AROMATICS

Substituents		*Nucleus**	$(S^\circ - \varepsilon^\circ A_S)$					
			$\varepsilon^\circ = 0.0$			$\varepsilon^\circ = 0.3$		
1	*2*		*Alum.*	*Sil.*	*Flor.***	*Alum.*	*Sil.*	*Flor.***
–H	–H	B	1.9	1.5	1.1	0.1	0.3	–0.1
		N	3.1	2.0	1.8	0.7	–0.3	0.3
–SR	–H	B	3.1	2.8	2.4	0.6	–0.1	0.8
		N	4.4	3.3	3.1	1.2	–0.1	1.1
–SR	–SR	B	4.5	4.1	3.7	1.2	0.1	1.6
		N	5.7	4.6	4.3	1.8	0.0	1.9
–OR	–H	B	3.6	3.3	2.9	1.2	0.2	0.9
		N	4.7	3.9	3.5	1.7	0.1	1.2
–OR	–OR	B	5.2	5.1	4.5	2.2	0.5	1.7
		N	6.4	5.6	5.2	2.8	0.4	2.1
–OR	–SR	B	4.8	4.6	4.1	1.6	0.3	2.3
		N	5.9	5.1	4.7	2.1	0.2	2.0
$-NO_2$	–H	B	4.6	4.3	4.2	2.1	0.3	1.9
		N	5.5	4.8	4.7	2.3	0.2	2.0
$-NO_2$	$-NO_2$	B	6.5	6.7	6.5	3.2	0.6	3.1
		N	7.4	7.2	7.1	3.5	0.5	3.3
$-NO_2$	–OR	B	5.9	5.9	5.6	2.7	0.6	2.5
		N	6.7	6.4	6.0	3.0	0.4	2.6
$-NO_2$	–SR	B	5.5	5.4	5.2	2.2	0.4	2.4
		N	6.4	5.9	5.7	2.5	0.3	2.6
$-CO_2R$	–H	B	5.2	5.0	4.5	2.5	0.9	2.5
		N	5.9	5.4	5.0	2.6	0.8	2.6
$-CO_2R$	$-CO_2R$	B	7.2	7.9	7.0	3.6	1.6	4.0
		N	7.9	8.3	7.6	3.7	1.4	4.2
$-CO_2R$	$-NO_2$	B	6.8	7.3	6.7	3.4	1.1	3.5
		N	7.6	7.7	7.2	3.5	0.9	3.7
$-CO_2R$	–OR	B	6.2	6.5	5.9	2.9	1.0	3.0
		N	7.0	7.0	6.5	3.1	0.8	3.2
$-CO_2R$	–SR	B	6.0	6.0	5.5	2.5	0.9	2.9
		N	6.7	6.5	6.0	2.7	0.8	3.0
–CHO	–H	B	5.2	5.4	5.0	3.1	1.4	2.5
		N	6.0	5.9	5.5	3.3	1.2	2.6
–CHO	–CHO	B	7.2	8.6	7.8	4.8	2.3	3.8
		N	8.0	9.1	8.3	5.0	2.2	4.0
–CHO	$-CO_2R$	B	7.2	8.2	7.4	4.2	1.9	4.0
		N	8.0	8.7	7.9	4.4	1.8	4.1
–CHO	$-NO_2$	B	6.9	7.7	7.2	4.0	1.5	3.5
		N	7.6	8.1	7.7	4.2	1.3	3.6
–CHO	–OR	B	6.3	6.9	6.3	3.6	1.5	2.9
		N	7.0	7.4	6.8	3.7	1.3	3.0
–CHO	–SR	B	6.0	6.5	6.2	3.2	1.3	3.0
		N	6.8	6.9	5.4	3.3	1.2	3.2

(continued on p. 507)

TABLE XIV (*continued*)

Substituents 1	Substituents 2	Nucleus*	$(S^\circ = \varepsilon^\circ A_s)$ $\varepsilon^\circ = 0.0$ Alum.	Sil.	Flor.**	$\varepsilon^\circ = 0.3$ Alum.	Sil	Flor.**
–COR	–H	B	5.6	6.2	5.4	3.2	1.6	2.7
		N	6.3	6.6	5.9	3.3	1.5	2.8
–COR	–COR	B	7.7	10.0	8.4	4.7	2.7	4.2
		N	8.4	10.5	8.9	4.8	2.6	4.3
–COR	–CHO	B	7.5	9.4	8.2	4.8	2.6	4.1
		N	8.2	9.8	8.7	4.9	2.4	4.2
–COR	$-CO_2R$	B	7.5	9.0	7.8	4.2	2.2	4.2
		N	8.2	9.5	8.3	4.3	2.1	4.3
–COR	–OR	B	6.6	7.7	6.7	3.6	1.7	3.2
		N	7.3	8.1	7.2	3.7	1.6	3.3
–COR	–SR	B	6.3	7.2	6.3	3.2	1.6	3.1
		N	7.0	7.7	6.8	3.3	1.5	3.3

* B, benzene derivatives; N, naphthalene derivative.
** × 0.63.

The elution of most samples of the above type will require eluents stronger than pentane, however, and for eluent strengths as large as 0.30 (as seen in Table XIV), alumina becomes a *better* adsorbent than silica or Florisil, because of the larger solute A_s values in the latter two adsorbents. Alternately, for separations of a substituted benzene from a similarly substituted naphthalene or higher aromatic, alumina is *always* preferable to silica, and comparable to Florisil, by virtue of the c_3 $(n - 6r)$ term of eqn. (3) for silica. Little difference in the separability of aromatic isomers on these three adsorbents appears indicated, since the few experimental q°_j values that have been obtained appear generally similar for each adsorbent.

The form of eqn. (3) is such that values of $(S^\circ - \varepsilon^\circ A_s)$ for additional solutes can be readily obtained from the data of Table XIV, using simple extrapolation. For example, $(S^\circ - \varepsilon^\circ A_s)$ for dinitro-methoxybenzene is simply the value for dinitrobenzene plus the difference between nitrobenzene and methoxy-nitrobenzene (*e.g.*, 7.8 for alumina with ε° equal 0.00). Such extrapolations must be carried out within the same group of solutes (grouped by lines in Table XIV). Similarly, the $(S^\circ - \varepsilon^\circ A_s)$ values for eluent strengths other than 0.00 and 0.30 can be obtained by simple interpolation or extrapolation between or beyond these latter ε° values.

With respect to the separation of the substituted aliphatics (III), these solute types tend to elute just before the corresponding substituted benzene on alumina (*e.g.*, S° values: alkyl ketones, 5.0, alkyl phenyl ketones; 5.6; alkyl sulfides, 2.7; phenyl alkyl sulfides 3.2)[3]. On silica, limited experimental data show the substituted aliphatics eluting with the substituted benzenes, but the differences in these two adsorbents in this respect are not very pronounced. Nothing is known about the relative separation of substituted aliphatics on Florisil, and it would be expected that silica and Florisil would be similar in this regard.

The separation of pyrrole derivatives (IV) such as indole and carbazole on these three adsorbents show some important differences. Relative to other sample types, the pyrroles are preferentially held on alumina, and tend to be eluted more readily from silica and Florisil. Previously summarized data suggest that this results from a

larger $Q°_i$ value for the pyrrole nitrogen group on alumina, and a smaller contribution to the effective area A_s of the solute. It has been suggested[6] that this difference between silica and alumina can be utilized in the separation of complex mixtures such as petroleum, where a preliminary segregation of the sample components is made over alumina, followed by reseparation of the alumina fractions over silica.

With respect to the separation of basic nitrogen types (V), chemisorption has been reported for these species on both silica and Florisil. Removal of free acid from the adsorbent *may* eliminate this effect in the case of silica, as discussed in the experimental section. As long as chemisorption exists, however, separations *within* the basic nitrogen class are not profitable, and the separation of the *total* basic nitrogen compounds from other sample components is facilitated. With the advent of ion-exchange techniques for the segregation of basic nitrogen compounds as a group from most samples of interest[6,19], the use of adsorption chromatography in this connection does not appear particularly desirable.

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GLOSSARY OF TERMS

A_s	solute surface volume, proportional to area required by solute on adsorbent surface
c_1, c_2, c_3	constants for particular adsorbents
$f(Q°_k)$, $L_1(Q°_i)$, $L_2(Q°_k)$	localization functions as defined in ref. 3 and Table III of this paper
n	number of aromatic carbon atoms in solute
$Q°_j$	solute group adsorption energy factor
$q°_j$	solute geometry factor
r	number of separate ring systems in solute (*e.g.*, one in naphthalene, two in dibenzyl)
$S°$	solute adsorption energy, pentane eluent
$\underline{R}°$	solute equivalent linear retention volume (ml/g)
V_a	adsorbent specific surface volume (ml/g), proportional to adsorbent surface area
α	adsorbent activity function
δ_i	contribution of solute group i to A_s, exclusive of localization effects
$\varepsilon°$	eluent strength function, proportional to eluent adsorption energy per unit area of adsorbent surface.

SUMMARY

The linear elution of several solutes by a number of eluents from Florisil samples of varying water content has been used to study this adsorbent in the context of preceding investigations on alumina and silica. Florisil exhibits certain unique

properties as an adsorbent. Many compounds tend to chemisorb on Florisil, and this restricts its usefulness in adsorption chromatographic separation. Relative to alumina and silica, the dependence of solute retention volume on Florisil water content is irregular when added water is less than 1 %. Both of these phenomena appear related to the presence of strong acid sites on the Florisil surface. In other respects, the adsorptive properties of $\geqslant$ 1 % H_2O-Florisil appear intermediate between those of silica and alumina. A general correlational equation for the prediction of retention volume in the chromatographic systems so far studied is given, along with a summary of specific references to the necessary experimental parameters for easy calculation. The unique separation capabilities of Florisil, alumina, and silica for the class of compounds so far studied are summarized and contrasted.

REFERENCES

1 L. R. SNYDER, *J. Chromatog.*, 6 (1961) 22.
2 L. R. SNYDER, *J. Chromatog.*, 8 (1962) 178.
3 L. R. SNYDER, *J. Chromatog.*, 8 (1962) 319.
4 L. R. SNYDER, *J. Chromatog.*, 11 (1963) 195.
5 L. R. SNYDER, *Anal. Chem.*, 33 (1961) 1527.
6 L. R. SNYDER AND B. E. BUELL, *Separation of Nitrogen, Sulfur, and Oxygen Compounds on Solid Adsorbents and Ion Exchange Resins*, presented at the *Symposium on Chemistry and Properties of the Petroleum-Type Sulfur and Nitrogen Compounds*, Univ. of Wyoming Science Camp, July 17–21, 1962.
7 J. R. SMITH, C. R. SMITH AND G. U. DINEEN, *Anal. Chem.*, 22 (1950) 867.
8 G. U. DINEEN, J. R. SMITH, R. A. VAN METER, C. S. ALLBRIGHT AND W. R. ANTHONY, *Anal. Chem.*, 27 (1955) 185.
9 H. J. CAHNMANN, *Anal. Chem.*, 27 (1955) 1235.
10 A. ASATOOR AND C. E. DALGLIESCH, *J. Chem. Soc.*, (1958) 1717.
11 Floridin Co., *Floridin Technical Data and Product Specifications*, Tallahassee, Fla., includes a detailed listing of other references to the use of Florisil in adsorption chromatography.
12 C. WALLING, *J. Am. Chem. Soc.*, 72 (1950) 1164.
13 J. S. BALL, private communication.
14 L. R. SNYDER, *Anal. Chem.*, 33 (1961) 1535.
15 L. R. SNYDER, *J. Phys. Chem.*, 67 (1963) 234.
16 L. R. SNYDER, *J. Phys. Chem.*, 67 (1963) 240.
17 L. R. SNYDER, *J. Phys. Chem.*, in press.
18 L. R. SNYDER, *J. Chromatog.*, 5 (1961) 468.
19 W. MUNDAY AND A. EAVES, *Proc. 5th World Petrol. Congr., New York*, 1959, Paper 9, Section V.

ZONE ELECTROPHORESIS IN COLUMNS OF AGAROSE SUSPENSIONS

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INTRODUCTION

Agar gel electrophoresis is a frequently used analytical tool, but is rarely used for preparative purposes, owing partly to the difficulty of localizing the zones in the gel without utilizing a denaturing staining technique, and partly to the rather cumbersome and tedious work required to elute the zones from the gel by repeated freezing and thawing. Both these disadvantages can be almost entirely eliminated, however, if a *suspension* of gel particles is utilized as a supporting medium instead of a coherent gel mass. As this electrophoresis method has been but briefly described before[1,2], it will now be treated in more detail. The experiments were originally made in suspensions of agar, but, owing to the many acidic groups in this polysaccharide, a series of undesirable phenomena arose. During a search for other more neutral anticonvection agents, it was found that agarose gave more satisfactory results. An important characteristic of agarose suspensions is that they show extremely low adsorption of the substances to be separated[1].

Owing to the characteristic flow properties of gel suspensions, the electrophoresis columns can be emptied with negligible zone spreading; this is a very important advantage of these systems.

MATERIAL AND METHODS

Preparation of agarose

For the preparation of agarose, a method based on that of ARAKI was originally used[1,3]. In addition, a simpler method, more suitable for larger scale preparations has been developed[4], based on the fact that acidic polysaccharides precipitate in the presence of quaternary ammonium compounds, leaving the neutral agarose in solution. This procedure also has the advantage that the product obtained has a higher gelling ability and can therefore be used in lower concentrations for the preparation of the gel suspension (see the next section). The agarose used in the experiments represented in Figs. 7 and 8 was prepared according to the new method; the other experiments were performed before this method was introduced.

Preparation of suspensions of agarose

The agarose is dissolved by boiling in a buffer volume somewhat larger than the volume of the electrophoresis tube (the same buffer is used as in the electrode vessels). The solution is allowed to cool without stirring in a stoppered Erlenmeyer flask at room temperature. The next day the gel is broken into small pieces by swirling the flask or

by gentle stirring; this agitation of the gel should proceed only for 1–2 min. If a film has formed on the surface of the gel, this film should first be removed. The agarose suspension should be de-aerated for some minutes before the electrophoresis tube is filled.

If the agarose is prepared according to ref. 4, the suspension will for most experiments have satisfactory anticonvection properties at concentrations as low as 0.16 % (0.16 g agarose per 100 ml buffer); if the original method[1] for preparation of agarose is followed a somewhat higher concentration is in general required (0.18 %). For determination of the minimum concentration of agarose required to stabilize the sample against convection, the following procedure can be used. A series of suspensions of different agarose concentrations (*e.g.* 0.13, 0.15, 0.17 and 0.19 %) is prepared and transferred to small test tubes. One drop of the sample solution (if uncolored it should be stained with a dye) is carefully added to each test tube. The lowest agarose concentration that prevents the sample from falling towards the bottom of the test tube, usually gives sufficient stabilization for electrophoresis, although a 0.01 % higher concentration is recommended.

Analytical procedures

The presence of agarose in the fractions obtained from the electrophoresis experiments is, in many analyses, not disturbing. Measurement of enzyme activities can thus often be carried out without removal of the gel particles[2]. Further, protein determinations by the FOLIN–LOWRY method[5] for preparation of electropherograms can be performed on aliquots of the collected, agarose-containing fractions. However, determinations of the material distribution by measurements of the ultraviolet absorption of the fractions give unreliable values owing to light scattering by the gel particles.

Removal of agarose

When it is essential to remove the agarose from the collected electrophoresis fractions, this can easily be done by centrifuging. For this purpose, simple table centrifuges can be used although larger centrifuges capable of giving 20,000 *g* are preferable. The carbohydrate concentration of the supernatant has been determined by the anthrone method[6] and was found to be 0.007 %, independent of the pH of the buffer from which the agarose suspension was prepared (only the pH interval within which most electrophoresis experiments are performed has been examined, *i.e.* 4.0–9.7). The degradation of agarose by hydrolysis is thus negligible, at least in the tested pH range.

If the gel pellet obtained after centrifuging is suspended in buffer and re-centrifuged and this washing procedure is repeated an almost 100 % yield is obtained.

ELECTROPHORESIS APPARATUS AND EXPERIMENTAL PROCEDURE

If the electrophoresis tube of an apparatus of conventional type[7] is filled with suspensions of commercial agar the suspension will move during electrophoresis towards the anode. If the anode is placed at the lower part of the electrophoresis tube, and this is closed with a dialysis membrane to prevent the easily movable suspension from running out of the tube, the suspension will be pressed towards the membrane, and the length of the agar column can be reduced as much as 50 %. The formation of cracks in the agar suspension has also been observed in such experiments. All these disturbing

effects are probably due to electroendosmosis, caused by the presence of charges in the agar, on the glass walls of the electrophoresis tube, and in the membrane.

In view of these difficulties, we studied the use of agarose instead of agar. It turned out that all the above disadvantages were considerably reduced when the suspension was prepared from this more neutral polysaccharide and the membrane was replaced by a plug of an agarose gel. A completely immobile agarose suspension was not obtained, however, until the electrophoresis tube was fitted with a "shunt" (S in Fig. 1). If this "shunt" is filled with buffer and the electrophoresis tube with agarose suspension, the hydrodynamic resistance in this "shunt" is lower than in the electrophoresis tube. The electroendosmotic reflow will consequently pass through the "shunt" and not through the electrophoresis tube. The suspension will therefore not be compressed. The existence of a hydrodynamic flow in the "shunt" can easily be demonstrated by adding a neutral dye, for instance DNP-ethanolamine[8], to the buffer in the "shunt". The direction of this flow can be reversed by reversing the direction of the current.

The method to be selected for removing the agarose bed from the electrophoresis tube depends upon the diameter of the column. As a result, the apparatus having a narrow electrophoresis tube has a few structural features, which are different from the larger equipment. Construction details (in some respects similar to those introduced by PORATH[7]) are given in the following sections. All operations which do not depend on the size of the electrophoresis tube are described only in section (a).

(a) Narrow columns (about 0.5 cm diam.)

A thin membrane D (Fig. 1), moistened with buffer, is fixed to the lower part of the electrophoresis apparatus by means of tightly stretched rubber bands R. As an alternative to the dialysis membrane, a plug N of a gel of agarose or polyacrylamide can be used. The agarose plug is prepared by sucking up a warm 2 % agarose solution through the porous polyethylene discs V 1 and V 2 and allowing it to cool. These discs will be firmly fixed to the glass tube if they are stamped to a diameter somewhat larger than the inner diameter of the tube. If a plug of polyacrylamide is used, this should have the composition $T = 5\%$, $C = 3\%$ (for the definition of these parameters and for the preparation of such a gel, see ref. 16).

The bottom section of the electrophoresis apparatus is filled up to the "shunt" S with agarose suspension and the electrophoresis tube E and the "shunt" S are filled with buffer. The screw clip B is closed and the buffer in the electrophoresis tube is sucked away and is replaced by agarose suspension*. The sample is applied and the rest of the electrophoresis tube is filled with agarose suspension as described below under "Application of the sample" (for these narrow columns it is, however, more convenient to replace the glass tube G in Fig. 4 with a piece of polyethylene tubing of 1 mm I.D.). The side arm A is attached and buffer from the electrode vessel is sucked up through the tubing T 1. It is essential that there are no air bubbles in the tubings T 2 and T 3. This can be controlled by switching on the current and observing whether or not it increases when the screw clip B is opened.

When the electrophoresis is finished and the column is to be emptied, the screw clip B is closed, the stopcock F is opened and the side arm A is detached. Starting from

* The use of a piece of polyethylene tubing for filling the electrophoresis tube will prevent air bubbles from forming in the agarose column.

the top of the electrophoresis tube, which is graduated in cm, 1-cm fractions are sucked out with the aid of a piece of polyethylene tubing (I.D. 1 mm). It is important that the free end of the tubing is kept *just* under the surface of the agarose suspension during this operation. Each 1-cm fraction is transferred to a test tube and aliquots are used for analyses.

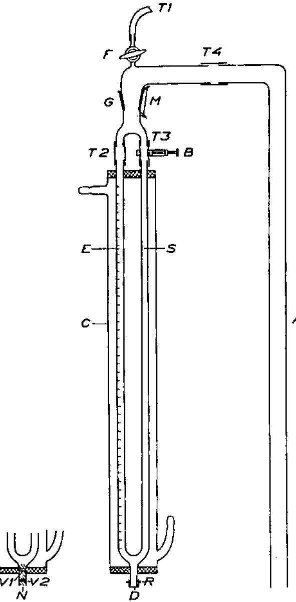


Fig. 1. Electrophoresis apparatus for narrow columns (diam. about 0.5 cm):

E = electrophoresis tube, graduated in cm
S = "shunt"
C = cooling mantle
A = side arm
D = dialysis membrane
R = rubber bands
B = screw clip
F = stopcock
G = ground glass joint
M = rubber bands
T1–T4 = polyvinyl chloride tubings

The dialysis membrane D can alternatively be replaced by a plug N of a gel of agarose or polyacrylamide fixed to the glass tube by means of the porous polyethylene[9] ("Vyon") discs V1 and V2, as shown in the drawing on the left.

(*b*) *Intermediate columns* (*about 0.9 cm diam.*)

These columns can be emptied in the same way as described above for narrow columns. However, the risk of remixing two adjacent zones during the sucking out procedure increases with the diameter of the column. It is therefore preferable to force the suspension out of the electrophoresis tube by pumping buffer into the top of it.

After the electrophoresis is finished, the screw clip B 1 (Fig. 2) is closed. A peristaltic pump is connected with the closed stopcock F. The screw clip B 2 is closed. The polyvinyl chloride tubing T 5 is cut up and the Y-tube Y is turned aside. During this procedure the tubing T 5 must be surrounded by the buffer in the electrode vessel, otherwise there is a risk that air bubbles will enter the electrophoresis tube and blurr the zones. For the same reason it is important that the apparatus is mounted so that the electrophoresis tube is not inclined. The electrode vessel is removed after its buffer

has been sucked away, the stopcock F is opened and the peristaltic pump is started. Flow rates of about 10 ml/h have been used for these intermediate columns.

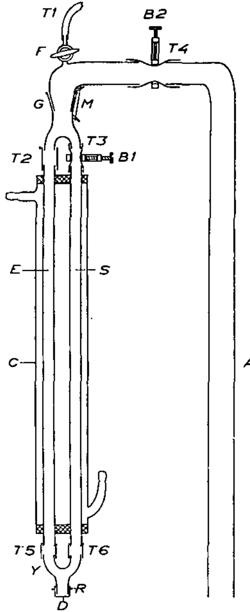


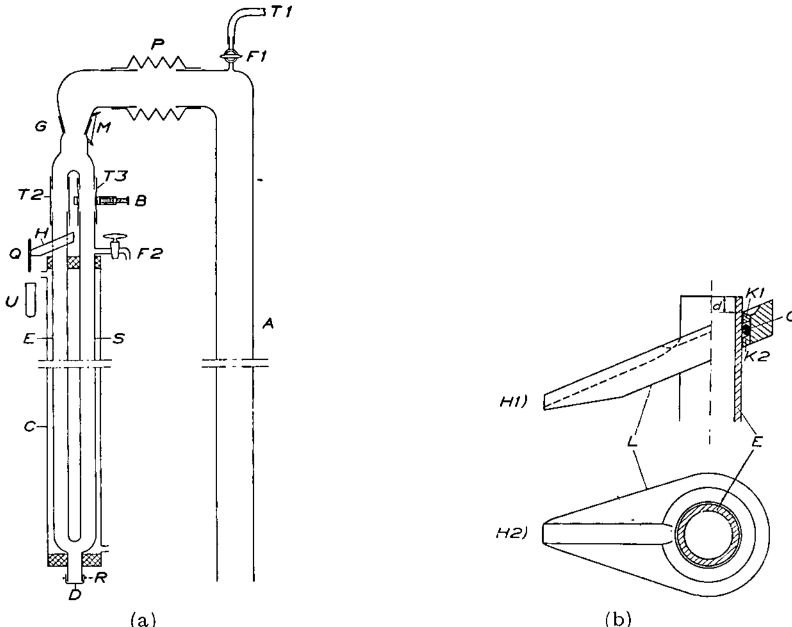
Fig. 2. Electrophoresis apparatus for intermediate columns (diam. about 0.9 cm):

E = electrophoresis tube
S = "shunt"
C = cooling mantle
A = side arm
D = dialysis membrane
R = rubber bands
B1, B2 = screw clips
F = stopcock
G = ground glass joint
M = rubber bands
Y = Y-tube
T1–T6 = polyvinyl chloride tubings

(c) Wide columns (about 2 cm diam.)

As described above, intermediate columns are emptied by pressing the agarose suspension downwards through the electrophoresis tube. A similar technique can be used for wider columns only if the outlet of the electrophoresis tube is modified, for instance by being supplied with a perforated disc (without this modification the suspension will run out of the electrophoresis tube as soon as the membrane has been removed and the outlet is no longer surrounded by buffer). When the zones pass the modified outlet, however, they will be distorted and so another method for emptying wide columns has been utilized:

When the electrophoresis is finished, the screw clip B (Fig. 3a) is closed. The stopcock F 1 is opened and the polyvinyl chloride tubing T2 is cut up and removed. The drainage device H is placed so that the distance d in Fig. 3 b is approximately 1 cm. The peristaltic pump is connected to the closed stopcock F 2. This is opened at the same time as the pump is started. The agarose suspension is forced up through the electrophoresis tube and runs down its walls into the drainage device H and drops into the test tubes U of a fraction collector via a glass rod Q.



(a) (b)

Fig. 3a. Electrophoresis apparatus for wide columns (diam. about 2 cm):

E = electrophoresis tube
S = "shunt"
C = cooling mantle
A = side arm
D = dialysis membrane
R = rubber bands
B = screw clip
F1, F2 = stopcocks
G = ground glass joint
M = rubber bands
T1–T3 = polyvinyl chloride tubings
P = corrugated perbunan tube[7]
H = drainage device (for details see Fig. 3b)
Q = glass rod
U = test tube in a fraction collector

Fig. 3b. Drainage device: H 1) = side view; the O-ring, O, lies in a track, formed by two obliquely cut perspex tubes K1 and K2, glued to the perspex plate L. The position of the drainage device on the electrophoresis tube E during the emptying procedure should be such that the distance d is about 1 cm. H 2) = top view.

(d) Application of the sample

About 3/4 of the water-cooled* section of the electrophoresis tube is filled with agarose suspension, as described above. The sample, previously dialyzed against the buffer, is diluted (1:1) with agarose suspension and sucked up into a syringe S via a glass tube G (Fig. 4). By turning the screw C the sample is carefully pressed out of the glass tube, which during the whole application procedure is slowly raised so that the bent orifice lies just in the surface of the suspension. It is important that the sample is evenly distributed across the agarose suspension, so that a sharp boundary is formed between the starting zone and the gel bed. The sample is covered by a 3-cm layer of agarose suspension in a manner similar to that used for the application of the sample. The remainder of the electrophoresis tube can then be more rapidly filled with suspension from a pipette.

* The temperature of the cooling water has in all experiments been 8–9°, except in that corresponding to Fig. 10, where it was 0.5°.

If the addition of agarose suspension involves an undesirable decrease in concentration of the sample, the application can be performed by injection instead of layering. In this case the glass tube G is lowered into the agarose column and the dia-

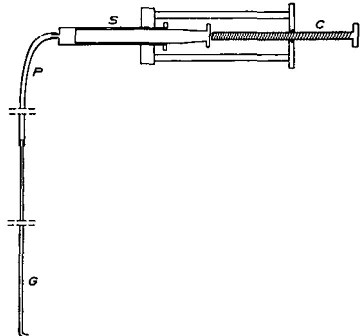


Fig. 4. Arrangement for application of the sample: S = syringe; P = polyethylene tubing; G = glass tube bent orifice; C = screw.

lysed sample (not mixed with agarose) is carefully pressed out and evenly distributed. Application of the sample by layering, is, however, preferable to that by injection.

As an exercise in application of the sample, the use of dyes, for instance pH-indicators, is recommended.

EXPERIMENTS AND RESULTS

Comparison between migration velocities in agarose suspension and in free solution

The migration velocities in 0.025 *M* sodium phosphate buffer, pH 7.3, of some proteins of different molecular size were determined in a free zone electrophoresis apparatus[10], where the diameter of the revolving uncooled electrophoresis tube was about 4 mm. These experiments were repeated in the same apparatus, with the only difference that the electrophoresis now was conducted in a 0.15 % agarose suspension, prepared in the same buffer. The current was the same in all experiments and equal to 2.5 mA. As the conductivities $\varkappa$ of the buffer alone and of the agarose suspension are different, the

TABLE I

COMPARISON BETWEEN MIGRATION VELOCITIES IN FREE SOLUTION AND IN AGAROSE SUSPENSION. Buffer: 0.025 *M* sodium phosphate, pH 7.3. The concentration of the agarose suspension prepared in the same buffer, was 0.15 %. The conductivity ratio of buffer solution to agarose suspension ($\varkappa_1/\varkappa_2$) was 1.05.

Protein	V_1 = *measured migration velocity in buffer solution (cm/sec · 10⁻⁴)*	V_2 = *measured migration velocity in agarose suspension (cm/sec · 10⁻⁴)*	$V_1\varkappa_1/V_2\varkappa_2$
R-phycoerythrin (mol.wt. 290,000)[11]	8.12	8.30	1.03
R-phycocyanin (mol.wt. 135,000)[11]	4.18	4.33	1.02
Human serum albumin (mol.wt. 68,000), stained with bromphenol blue	7.16	6.67	1.13
Cytochrome *c* from horse heart (mol.wt. 13,000)	3.58	3.76	1.00

measured migration velocities V_1 and V_2 (see Table I) should be transformed to those in the unit field to be comparable. At constant current the latter velocities are proportional to $V \cdot \varkappa$. The ratio between these in free solution and in agarose suspension ($V_1\varkappa_1/V_2\varkappa_2$) is given in the table.

Zone broadening during emptying of the column

This experiment was carried out with an electrophoresis apparatus of an older type with a detachable, not water-cooled "shunt". The dimensions of the electrophoresis tube were 0.9 × 50 cm*. The supporting medium was a 0.16 % agarose suspension, prepared in sodium acetate buffer (pH 4.85, ionic strength 0.01), to which calcium chloride was added to a concentration of 0.005 *M*. Two colored zones were applied, one containing a protein, R-phycoerythrin[11], and the other a cell suspension of *Chlorella pyrenoidosa*. The column was emptied by pumping buffer into the top of the electrophoresis tube, as described previously for intermediate columns. The flow rate was adjusted to 10 ml/h**. From the photographs in Fig. 5, taken at different intervals during the emptying, it is evident that this procedure does not cause a detectable broadening of the zones. In this experiment, the zone or *Chlorella* was introduced in order to have a zone in which the broadening due to diffusion would be negiglible.

Electrophoresis of low molecular weight substances

The same electrophoresis apparatus was used as in the previous experiment. The composition of the supporting medium was also the same. About 1 mg each of DNP-aspartic acid, DNP-serine and ε-DNP-lysine was added to 2 ml agarose suspension, which was then heated to boiling. After cooling, 0.4 ml of this sample was applied. A zone of the neutral marker substance DNP-ethanolamine[8] was introduced some centimeters above the sample zone. The run was performed at 6 mA. After 3 hours' electrophoresis the DNP-amino acids were well separated and the emptying procedure started (details are as described above for intermediate columns). Fractions of 0.5 ml were collected and were diluted to 1.0 ml before being submitted to spectrophotometric analysis at 360 mμ in a 1-cm cuvette. The electropherogram is seen in Fig. 6a. The photograph in Fig. 6b was taken just before the column was emptied.

Electrophoresis of proteins

(*a*) *Fractionation of serum on a 0.5-cm column*. The electrophoresis tube (0.5 × 55 cm, Fig. 1) was filled with a 0.17 % agarose suspension, prepared in sodium veronal buffer, pH 8.6, ionic strength 0.05. About 75 μl dialyzed normal human serum was mixed with an equal volume of the agarose suspension and applied at a distance of 10 cm from the top of the cooling mantle. After 17 hours' electrophoresis at a current of 5.5 mA the column was emptied, as described above for narrow columns, by sucking out 1-cm fractions, (*i.e.* about 180 μl). Aliquots of 100 μl were used for protein determination according to the method of FOLIN AND LOWRY[5]. The electropherogram is given in Fig. 7.

(*b*) *Fractionation of serum on a 2.0-cm column*. The above experiment was repeated with an apparatus, in which the electrophoresis tube had the dimensions

* By the length of an electrophoresis tube we mean throughout this paper the length of the water-cooled section.

** Higher pump speeds can be tolerated, however. The maximal emptying rate that can be used without causing distortion of the zones varies with the agarose concentration, but can easily be determined by introducing a zone containing a dye.

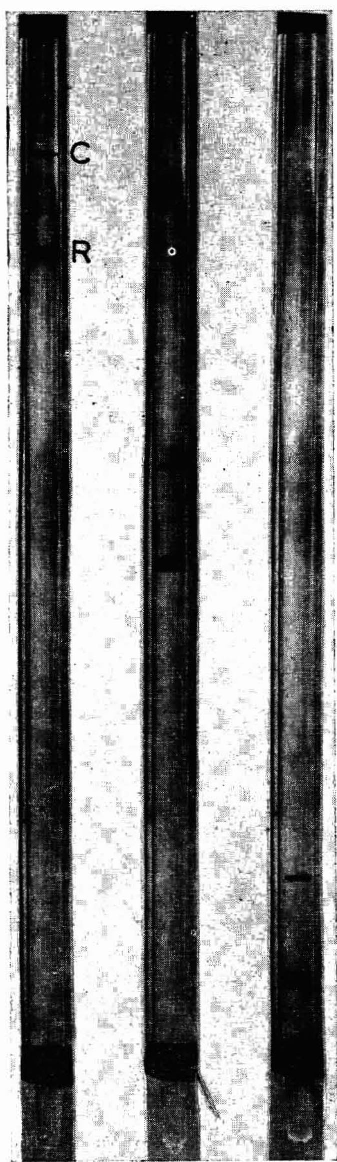
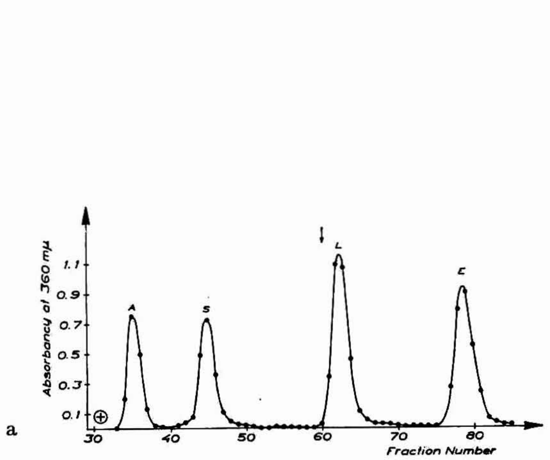


Fig. 5. Photographs, illustrating that the emptying procedure does not cause a detectable broadening of the zones. The zones C and R contain *Chlorella pyrenoidosa* and R-phycoerythrin, respectively. The column, of dimensions 0.9 × 50 cm, was emptied at a rate of 10 ml/h.



a

b

Fig. 6. (a) Electrophoresis in a 0.16 % agarose suspension of a mixture of DNP-aspartic acid (A), DNP-serine (S) and ε-DNP-lysine (L). Electrophoresis tube dimensions: 0.9 × 50 cm. Buffer: sodium acetate, pH 4.85, ionic strength 0.01, + 0.005 *M* calcium chloride. Sample: 0.4 ml agarose suspension containing 0.2 mg of each DNP-amino acid. Current: 6 mA. Duration: 3 h. Emptying rate: 10 ml/h. The place for application of the sample is indicated by an arrow. As a neutral marker, DNP-ethanolamine (E) was applied at fraction 76. (b) A photograph of the separation in Fig. 6a. The column is of an older type with an outer "shunt", which has to be detached before the empyting and is therefore not seen in the photograph.

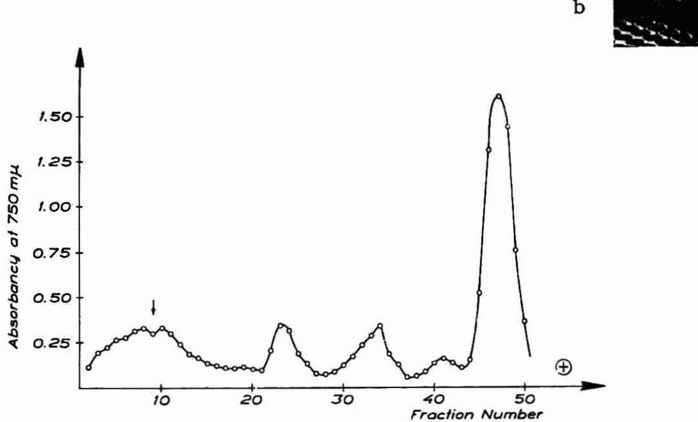


Fig. 7. Electrophoresis in a 0.17 % agarose suspension of normal human serum. Electrophoresis tube dimensions: 0.5 × 55 cm. Buffer: sodium veronal, pH 8.6, ionic strength 0.05. Sample: 75 μl serum + 75 μl agarose suspension. Current: 5.5 mA. Duration: 17 h. The column was emptied by sucking out 1-cm fractions. The arrow at fraction number 9 indicates the position of the starting zone. The protein contents were determined by absorption measurements at 750 mμ according to the method developed by FOLIN–LOWRY[5].

2.0 × 85 cm (Fig. 3a). About 2 ml dialyzed normal human serum was diluted (1:1) with the agarose suspension and applied 22 cm from the top of the cooling mantle. The current was adjusted to 50 mA. The experiment was interrupted after 62 h when the yellow albumin zone was 20 cm from the bottom of the cooling mantle. The column was emptied at a pump rate of 60 ml/h (the technique is as described for wide columns). 100 μl of each 2-ml fraction was used for the determination of the protein concentration according to the FOLIN–LOWRY method[5]. The six peaks (Fig. 8) were analyzed by paper electrophoresis, and, as expected, they contained prealbumin, albumin, α_1-globulin, α_2-globulin, β-globulin, and γ-globulin. In these analyses, a zone-sharpening method[12] was used, which allows dilute protein samples to be applied directly to the paper without previous concentration (owing to electroendosmosis the γ-globulin did not reach the dialysis bag and therefore could not be zone sharpened by this method). The result is given in Fig. 9. The spot corresponding to prealbumin was poorly reproduced in the photograph and has therefore been omitted. The paper electrophoresis was conducted in the same buffer as the agarose electrophoresis.

Electrophoresis of subcellular particles

These experiments were performed before the agarose was introduced as an anti-convection agent and before the electrophoresis apparatus was fitted with a "shunt". The agar* column was therefore compressed during the electrophoresis (the position of the starting zone is therefore not indicated in Figs. 10 and 11). Comparatively few cracks were, however, observed, which may be due to the fact that the buffer used contained magnesium ions**.

(*a*) *Electrophoresis of a microsome preparation from rat brain.* Some preliminary experiments on microsomes from rat brain were carried out in collaboration with DR. TOSCHI***. The microsomes were prepared as described by him[13]. About 0.75 ml of the sample, which contained around 5 mg protein per ml, was mixed with an equal volume of agar suspension and applied about 15 cm from the top of the electrophoresis tube, which had the dimensions 0.9 × 60 cm. The temperature of the circulating cooling water was +0.5°. The electrophoresis was conducted for 12 h at 10 mA in a sodium borate buffer, pH 8.2, ionic strength 0.03, containing 0.0083 *M* sodium sulfate and 0.001 *M* magnesium sulfate. The column was emptied as described previously for intermediate columns. Spectrophotometric measurements at 260 mμ in a 1-cm cuvette of 1.5-ml fractions gave the electropherogram in Fig. 10. The ribonucleic acid (RNA) and the protein contents were determined in the two main fractions A and B[13]. Although variable results were obtained with different microsome preparations, the RNA concentration (on protein basis) was always higher in the faster migrating zone A. The highest value observed in this zone was about 250 μg RNA per mg protein, while zone B in the same experiment contained about 20 μg RNA per mg protein. As shown by HANZON AND TOSCHI[14] with the aid of electron microscopy, brain microsomes consist of two submicroscopic structures, vesicles formed by thin membranes and small dense

* 0.14% suspensions of Difco Noble Agar were used.

** When an electrophoresis is performed at potential gradients as high as 40 V/cm, especially in buffers of low ionic strength, cracks may sometimes occur also in agarose suspensions. In such cases the tendency for crack formation can be decreased if a plug N of a gel of agarose or polyacrylamide is used instead of a dialysis membrane D (Fig. 1) and if magnesium ions are added to the buffer, for instance in the form of magnesium chloride, to a concentration of 0.004 *M* (for preparation of these plugs, see the section "Narrow columns").

*** Present address: Istituto Superiore di Sanità, Rome, Italy.

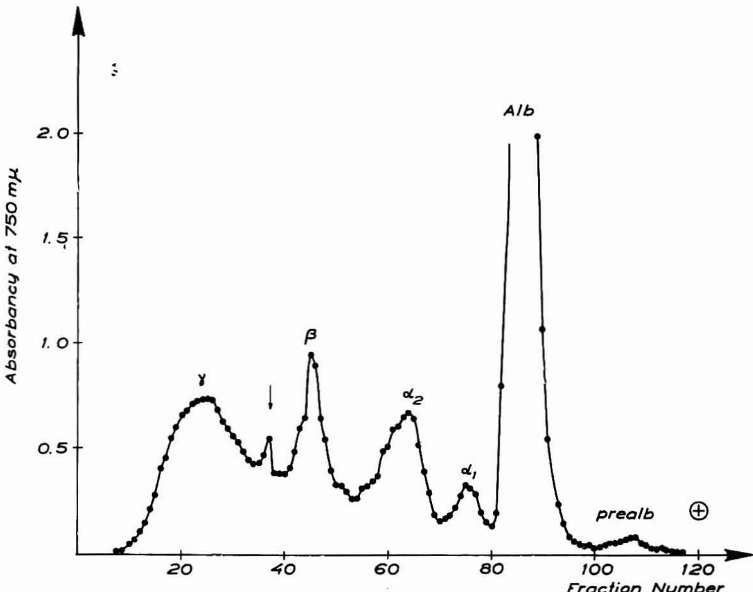


Fig. 8. Electrophoresis of normal human serum. Agarose concentration and buffer composition were the same as those given in the legend to Fig. 7. Electrophoresis tube dimensions: 2.0 × 85 cm. Sample: 2 ml serum + 2 ml agarose suspension. Current: 50 mA. Duration: 62 h. Emptying rate: 60 ml/h. The arrow at fraction number 37 indicates the position of the starting zone.

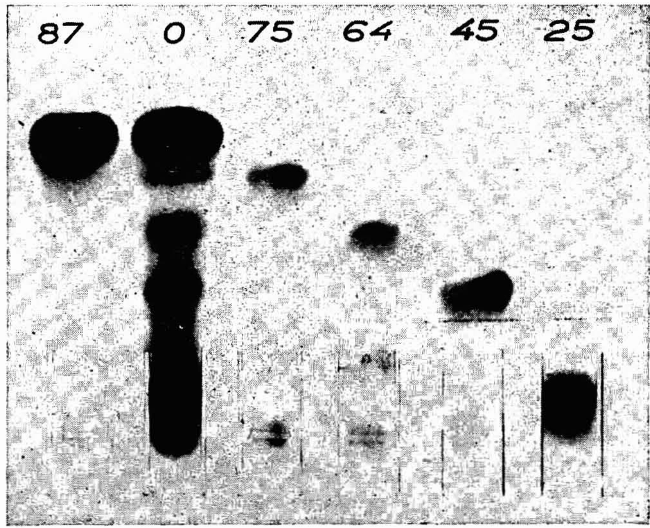


Fig. 9. Paper electrophoresis of the material in the main peaks in Fig. 8, *i.e.* fractions 25, 45, 64, 75 and 87. The pattern indicated by O was obtained from unfractionated serum. By using a zone-sharpening method[12] no preconcentration of the fractions was necessary, except for γ-globulin.

particles, RNA being associated with the particles. Electronmicroscopic examination of samples from the zones A and B showed a definite separation of the particles, which collected in zone A in high concentration.

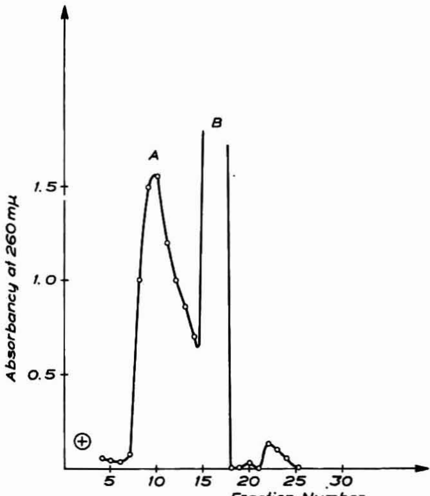


Fig. 10. Electrophoresis in a 0.14% agar suspension of a microsome preparation from rat brain. Electrophoresis tube dimensions: 0.9 × 60 cm. Buffer: sodium borate, pH 8.2, ionic strength 0.03, + 0.0083 *M* sodium sulfate + 0.001 *M* magnesium sulfate. Sample: 0.75 ml of the microsome preparation + 0.75 ml agar suspension. Current: 10 mA. Duration: 12 h. Emptying rate: 10 ml/h. The fastest migrating zone A has a much higher RNA concentration (on protein basis) than zone B. The dense particles[14] were highly enriched in zone A.

(*b*) *Electrophoresis of a microsome preparation from baker's yeast.* This experiment was carried out in collaboration with Dr. LEVIN in this Institute. The microsomes were prepared essentially as described by CHAO AND SCHACHMAN[15]. The microsomes were suspended in a solution, 0.0125 *M* with respect to potassium phosphate, pH 7.45, and 10^{-3} *M* with respect to magnesium sulfate. For preparation of an agar suspension of this ionic composition, the magnesium sulfate must be added to the phosphate buffer after the agar suspension has cooled to prevent precipitation. No agar suspension was added to the sample and therefore it was not layered but injected into the agar column as described under "Application of the sample". The volume of the microsome solution was about 2.5 ml and it was injected 15 cm from the top of the cooling mantle. The dimensions of the electrophoresis tube were 2.0 × 85 cm. The experiment was performed at 20 mA for 23 h. After some hours' electrophoresis three opalescent zones could be observed. The fastest of these, however, became gradually so faint that it could no longer be detected. The column was emptied at a rate of 2 ml/min, with the aid of the drainage device shown in Fig. 3 b. The different fractions were analyzed by absorption measurements at 260 and 280 mμ in a 1-cm cuvette. An estimation of the protein contents were made also by the FOLIN–LOWRY method[5]. The result of these analyses is given in the electropherogram in Fig. 11, which shows that the main peak I is followed by a peak II, somewhat richer in protein. The slowest migrating zone, IV, was most opalescent; its protein contents must be very low to judge from the Folin-curve.

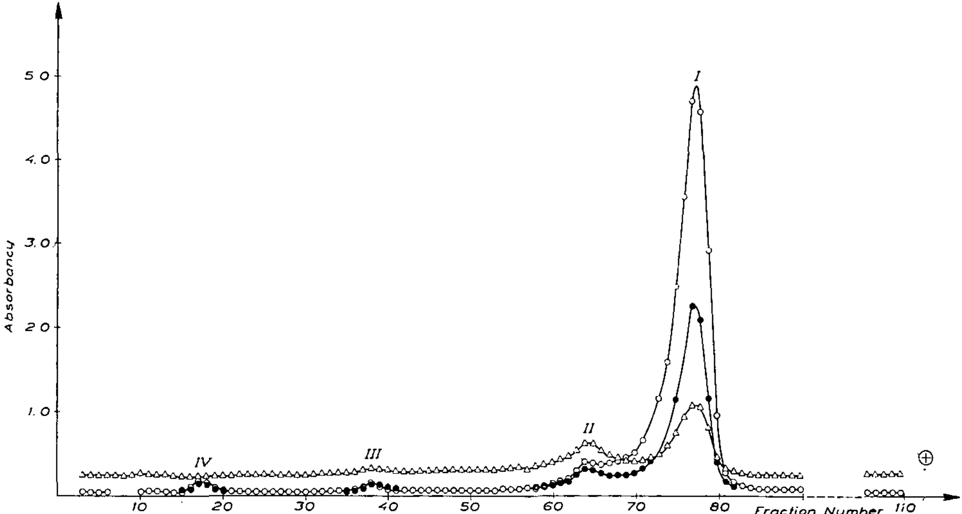


Fig. 11. Electrophoresis in a 0.14 % agar suspension of a microsome preparation from baker's yeast. Electrophoresis tube dimensions: 2.0 × 85 cm. Buffer: 0.0125 *M* potassium phosphate, pH 7.45, + 0.001 *M* magnesium sulfate. Sample: 2.5 ml of the microsome preparation. Current: 20 mA. Duration: 23 h. Emptying rate: 120 ml/h. Open and solid circles correspond to absorption measurements at 260 and 280 mμ, respectively. The protein contents (triangles) were determined at 750 mμ according to the method of FOLIN–LOWRY[5]. Some microsome preparations contain more material in peaks II, III and IV, and sometimes a smaller peak is obtained in front of peak I.

DISCUSSION

Agarose suspensions provide good stabilization against convection even at concentrations as low as 0.15 %. It is therefore not surprising that the electrophoretic migration velocities of proteins in these suspensions are similar to those in free solution (Table I). Each preparative agarose electrophoresis experiment can therefore be utilized for approximate determinations of mobilities as well, provided a neutral reference substance is used to correct for electroendosmosis[8], which may be rather pronounced (Figs. 6 a and 7). The fact that the "sieving effect" is very low in agarose suspensions is in accordance with the finding by ACKERS AND STEERE[17] that in a 0.15 % agar gel the diffusion coefficients of hemoglobin and southern bean mosaic virus agree within a few percent with the values obtained in free solution. The pore size of the gel matrix thus allows migration of much larger cell particles than the microsomes used in the above experiments (Figs. 10 and 11). It should be pointed out, however, that when the particle size is extended to whole cells, like bacteria, the sample remains at the place of application.

Electrophoresis in agarose suspensions often gives very sharp separations. Fig. 8 may serve as an example. As seen, α_1-globulin in human serum can easily be isolated from albumin, a separation not so easily obtained in preparative zone electrophoresis. Another example is Fig. 6 in ref. 2, which shows that the major part of the enzyme activities after the run was found in a volume only three times larger than that of the applied sample. The creation of such narrow zones is favored by the homogeneity of the agarose columns, as well as by the extremely low tendency to adsorption[1]. A special advantage is also the fact that the emptying procedure does not cause a

detectable distortion of the zones (Fig. 5), as suspensions of agarose move as a rigid body or piston when forced through the electrophoresis tube ("plug flow").

Before starting experiments on a large column it may save time and material to make preliminary experiments on a small column in order to ascertain which buffer, pH, and ionic strength etc. will give the best resolution. This requires the result obtained with the small column to be similar to that obtained with the large one. That this is the case when agarose suspensions are used as the supporting medium is evident from a comparison between Figs. 7 and 8.

As the agarose columns are semi-transparent, the highest potential gradient that may be used without the zones being distorted can in each experiment be determined easily by observing the shape of the sample zone. If this is not visually detectable, a zone of colored protein is introduced (for this purpose low molecular weight substances should not be used, because they have a strong diffusion which will be superimposed on the above distortion and make its detection more difficult). When the instability of the sample requires short duration of the experiment one may be forced to use an extremely high potential gradient. The resulting curvature of the zones gives rise to a decrease in the resolution. If necessary this can, however, be considerably increased by forcing agarose suspension into the electrophoresis tube in a direction opposite to that of the migration of the zones. If such a hydrodynamic compensation is made at a suitable rate a curved zone can be restored to its original flat shape.

As further examples of proteins being purified by the electrophoresis method described in this paper, uridine kinase from Ehrlich ascites tumor[18], uridine and deoxyuridine phosphorylases from the same source[19], and human growth hormone[20] can be mentioned.

We have earlier pointed out that agarose has some advantages over agar as an anticonvection agent[1,21]. This has now been confirmed by GHEȚIE AND MOȚET-GRIGORAȘ[22] who found that in electrophoresis of human serum, agarose gels give about twice as many zones as agar gels. The same authors also claim that a more easily interpretable pattern of immunoelectrophoretic precipitation lines is obtained with agarose.

A great number of electrophoresis methods are now available for purification of substances of biological origin. It is beyond the scope of this paper to discuss all their advantages and disadvantages, but a brief comparison might be appropriate.

An ideal anticonvection agent should not, in general, show any tendency to adsorb the substances to be separated. In this respect columns of agarose suspensions are preferable to those of starch grains or cellulose powder. Plastic powders, for instance Pevikon, are also characterized by a low adsorption, but unfortunately they are not particularly efficient as anticonvection media[2]. The broadening of a zone during the elution or emptying procedure is of great interest when discussing different electrophoresis methods. Displacement of the zones from a column by a hydrodynamic buffer flow involves an inevitable increase in their width. As pointed out previously, however, the emptying technique used for agarose columns causes a negligible broadening of the zones. Another factor that must be taken into account in these comparisons is the degree of contamination of the bed material with the isolated fractions. In this respect Pevikon is superior to agarose (as mentioned, the concentration of agarose in the supernatant after centrifugation is about 0.007 %); cellulose and starch take an intermediate place. In some cases the presence of agarose is a disturbing factor. Thus,

in determinations of the carbohydrate content of a protein, agarose electrophoresis should not be used at the last step in a series of purification methods. The traces of agarose in the supernatant can, of course, be removed by electrophoresis or chromatography on inert columns (owing to the neutral character of agarose it is easy to establish the experimental conditions for its removing).

The bed materials discussed here give electropherograms which resemble those obtained in free electrophoresis. During recent years gels of starch and polyacrylamide have been introduced, which, however, give electropherograms of a totally different appearance owing to the "molecular-sieving" action of these gels. If a sample is submitted to two consecutive electrophoresis runs, one in a medium without and the other in a medium with "molecular-sieving" properties, a high grade of purification can be expected. Electrophoretic "molecular-sieving" has frequently been used for analytical purposes. In its present form it can be utilized only for preparative purposes on a mg scale[23]. The difficulty of eluting the zones without distortion increases with the cross-section area of the gel. If electrophoretic "molecular-sieving" is to be developed as a large scale preparative method the elution technique must be refined.

ACKNOWLEDGEMENTS

I am indebted to Professor ARNE TISELIUS for extremely valuable discussions. Part of the experimental work has been performed by Miss ELSY NOLANDER, which is gratefully acknowledged. This investigation has been supported by grants from the National Science Foundation (grant number G-18702 to Prof. ARNE TISELIUS).

SUMMARY

Suspensions of gel particles of agarose can be used as supporting media for electrophoresis on an analytical as well as a preparative scale for purification of low molecular weight substances, proteins, and also of subcellular particles, such as microsomes. Particular advantages are that the adsorption to the agarose of the substances to be separated is extremely low and that the columns can be emptied with negligible distortion of the zones. The agarose is removed by centrifugation. As the agarose concentration can be kept as low as 0.16%, the suspensions are semitransparent, and so colored and opalescent zones can be detected comparatively easily. The electrophoretic migration velocities of proteins in these suspensions are similar to those in free solution.

REFERENCES

1 S. HJERTÉN, *Biochim. Biophys. Acta*, 53 (1961) 514.
2 J. PORATH AND S. HJERTÉN, IN D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 9, Interscience, New York, 1962.
3 C. ARAKI, *J. Chem. Soc. Japan*, 58 (1937) 1338.
4 S. HJERTÉN, *Biochim. Biophys. Acta*, 62 (1962) 445.
5 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
6 T. A. SCOTT AND E. H. MELVIN, *Anal. Chem.*, 25 (1953) 1656.
7 J. PORATH, *Biochim. Biophys. Acta*, 22 (1956) 151.
8 D. L. MOULD AND R. L. M. SYNGE, *Biochem. J.*, 58 (1954) 585.
9 J. PORATH AND K. STÖRIKO, *J. Chromatog.*, 7 (1962) 385.
10 S. HJERTÉN, *Protides Biol. Fluids, Proc. 7th Colloq., Bruges, 1959*, Elsevier, Amsterdam, 1960, p. 28.

[11] H. Kylin, *Z. Physiol. Chem.*, 69 (1910) 169.
[12] S. Hjertén, *Biochim. Biophys. Acta*, 32 (1959) 531.
[13] G. Toschi, *Exptl. Cell Res.*, 16 (1959) 232.
[14] V. Hanzon and G. Toschi, *Exptl. Cell Res.*, 16 (1959) 256.
[15] Fu-Chuan Chao and H. K. Schachman, *Arch. Biochem. Biophys.*, 61 (1956) 220.
[16] S. Hjertén, *Arch. Biochem. Biophys.*, Suppl. I (1962) 147.
[17] G. K. Ackers and R. L. Steere, *Nature*, 192 (1961) 436.
[18] O. Sköld, *J. Biol. Chem.*, 235 (1960) 3273.
[19] H. Pontis, G. Degerstedt and P. Reichard, *Biochim. Biophys. Acta*, 51 (1961) 138.
[20] P. Roos, H. R. Fevold and C. A. Gemzell, *Biochim. Biophys. Acta*, 74 (1963) 525.
[21] S. Brishammar, S. Hjertén and B. v. Hofsten, *Biochim. Biophys. Acta*, 53 (1961) 518.
[22] V. Gheţie and D. Moţet-Grigoraş, *Acad. Rep. Populare Romine, Studii Cercetari Biochim.* 3 (1962) 409.
[23] S. Hjertén, *J. Chromatog.*, 11 (1963) 66.

CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART IX. A QUANTITATIVE STUDY OF THE HYDROLYSIS OF THE TETRAMETAPHOSPHIMATE ANION

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Recent investigations[1], into the hydrolytic behaviour of the tetrametaphosphimate (TeMPm) anion, have indicated the unusual ring degradation upon acid hydrolysis, yielding the di-imidotrimetaphosphate (DITMP) anion, together with orthophosphate (MP).

$$\text{TeMPm} \xrightarrow{H_3O^+} \text{DITMP} + \text{MP} + NH_4^+ \qquad (1)$$

The di-imidotrimetaphosphimate undergoes further hydrolysis to mono-imidotrimetaphosphimate (ITMP), and thence to trimetaphosphate (TMP), pyrophosphate (DP), and orthophosphate very slowly as described in earlier papers[1-5].

With improved methods of anion-exchange chromatography, it has been possible to study the hydrolysis of TeMPm quantitatively, and show that the eight-membered phosphimate ring is more stable to acid hydrolysis than its six-membered analogues.

EXPERIMENTAL

Preparation of ammonium tetrametaphosphimate

Investigations of the salts of TeMPm by STOKES[6], showed that the tetra-sodium potassium and silver salts, as well as the acid itself, were all highly insoluble in water. However, the ammonium salt is fairly soluble, and hence is the only salt suitable for hydrolytic studies. The behaviour and stoichiometry of these salts have been investigated by STEGER AND LUNKWITZ[7,8].

10 g of pure tetraphosphonitrilic chloride $(PNCl_2)_4$ (Albright & Wilson Ltd.), were dissolved in 200 ml of dioxan, and mixed with a solution of 40 g of ammonium acetate dissolved in 40 ml of water. The mixture was then stirred continuously for 90 h. The white precipitate was separated by filtration, and washed with methanol. Pure

ammonium tetrametaphosphimate was recovered from an aqueous solution by dissolution of this white product and precipitation with methanol. The yields were always of the order of 55 %; elemental analysis, together with infra-red, paper and anion-exchange chromatographic techniques, showed the product to be pure.

Analysis of hydrolytic products

To study quantitatively the hydrolytic breakdown of TeMPm to DITMP, ITMP, and MP, by anion-exchange chromatography, it is necessary to achieve good separations between the four anions concerned. However, it was found that using the standard 50 × 0.9 cm column packed with 100–200 mesh size Dowex-1 X 8 resin, and eluting with 0.075 *M*–0.75 *M* potassium chloride buffered to pH 5.0,[5] that TeMPm and DITMP have almost identical retention volumes (Fig. 1a). Experimentation with different gradient chloride eluants proved of little value in separating the two anions,

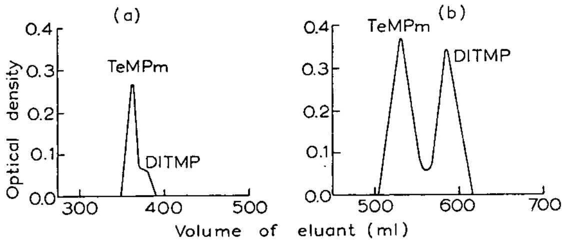

Fig. 1. Elution patterns of TeMPm and DITMP using differing columns and gradient elution conditions. (a) 0.075–0.75 *M* KCl solutions. (b) 0.050–0.80 *M* KCl solutions.

and thus this type of column geometry was abandoned. In order to overcome such difficulties, it was decided to use a column where the two anions might separate whilst exchanging slowly down a long column containing more bed volume of resin per unit column length than that previously described. With this in mind, a column 100 cm × 1.2 cm diameter of Dowex-1 X 8, 100–200 mesh resin was used, with a chloride gradient eluant of 0.05 *M*–0.80 *M* potassium chloride solution. This gradient took a greater volume of eluant to reach the optimum value of desorption (see ref. 5), but

TABLE I

Summary of column conditions

Column: 100 cm × 1.2 cm diameter
Resin: Dowex-1 X 8, 100–200 mesh size
Eluants: 0.05 *M* KCl–0.80 *M* KCl buffered to pH 5.0
Flow rate: 35–40 ml/h

Retention volumes	*(ml)*
Orthophosphate	220
Trimetaphosphimate	420
Tetrametaphosphimate	520
Diimidotrimetaphosphate	580
Imidotrimetaphosphate	780
Trimetaphosphate	1100

caused sharper elution peaks, because the d[Cl⁻]/dt was greater. Such a gradient prevented broadening of peaks, and hence less error in evaluating absolute recoveries for kinetic measurements. The separation achieved is shown in Fig. 1b.

The separation achieved using the conditions mentioned above, was not improved upon markedly by experimentation using other differing gradients, and so was used for studying the hydrolysis of TeMPm (Table I).

Study of hydrolysis

Approximately 0.5 g of ammonium TeMPm were dissolved in 50 ml of acetate buffer pH 3.62 in a 100 ml graduated flask, which was placed in a thermostatically controlled water-bath at the required temperature. 5.0 ml samples were removed from the solution at noted time intervals, and the reaction stopped by pouring the samples

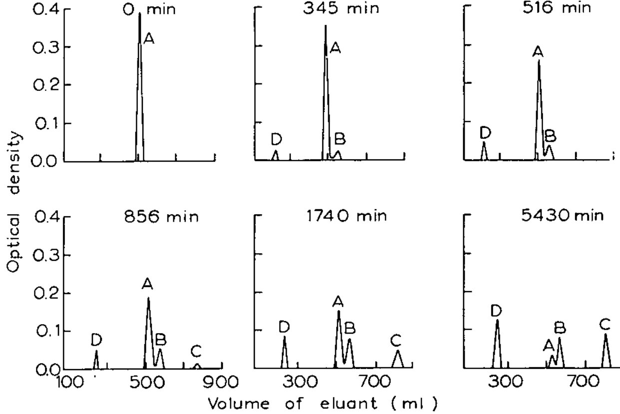

Fig. 2. Elution patterns for the hydrolysis of TeMPm at 65° and pH 3.62. A = TeMPm; B = DITMP; C = ITMP; D = MP.

into 5 ml of ice-cold 0.2 N caustic soda solution. 1 ml of this solution was absorbed on to the anion-exchange column, eluted and the phosphorus-containing fractions estimated in the manner as previously described[5]. The results of analysis at the various time intervals are shown in Fig. 2, and typical results are given in Table II, where k_0

TABLE II

Temperature 65.0°		*Temperature 80.0°*	
Time of hydrolysis (min)	$k_0 \times 10^4$ *(min⁻¹)*	*Time of hydrolysis (min)*	$k_0 \times 10^3$ *(min⁻¹)*
345	3.63	355	1.35
516	3.75	634	1.31
856	3.78	879	1.32
1,268	3.65		
1,748	3.50		

is velocity constant of overall breakdown of TeMPm, which was calculated in the following manner.

Each phosphate species recovered by the anion-exchange separation is oxidatively hydrolysed to orthophosphate by heating with concentrated nitric acid, and then estimating the orthophosphate as the ammonium phosphovanadomolybdate complex. Thus we have the analytical recovery procedure:

$$\text{TeMPm} \longrightarrow \text{MP} + \text{DITMP} \longrightarrow \text{ITMP} \not\longrightarrow \text{TMP}$$

$$\text{ITMP} \not\searrow \text{TP} + \text{DP} + \text{MP}$$

4 × orthophosphate (by oxidation process)	⟶	1 × orthophosphate (recovered as orthophosphate)	+	3 × orthophosphate (by oxidation from) DITMP and ITMP)

—|→ indicates reaction sequences which were not detected

It would be expected that the recovery of MP would be one-third that of the combined recoveries of DITMP and ITMP, provided that reaction (1) is followed. As reported previously[1], the recovery of MP always exceeds that of one-third of total recovery of DITMP + ITMP, which most probably indicates the partial breakdown of TeMPm to chain imido-phosphates which, in turn, immediately hydrolyses under the hot acid conditions, to MP. It has been proved[5] that DITMP and ITMP decompose only very slowly to chain phosphates, hence the discrepancy in the analytical figures is directly attributable to the hydrolysis of TeMPm to chain species. Therefore k_0 does not express the true reaction constant for the breakdown of TeMPm to DITMP, since it incorporates the reaction constant for ring fission to chain phosphates. To evaluate k^1, the rate constant for ring degradation, it is necessary to sum the total recovery of DITMP + ITMP, and take one-third of this value as the concentration of orthophosphate resultant from ring degradation. If one uses this factor as x in the equation:

$$k^1 = \frac{1}{t} \ln \frac{a}{a - x}$$

where a is the total recovery of phosphorus, which at time $t = 0$, is all TeMPm, then the variation of ln $a/(a - x)$ *versus* time is shown in Fig. 3, and the values of k^1 are given in Table III.

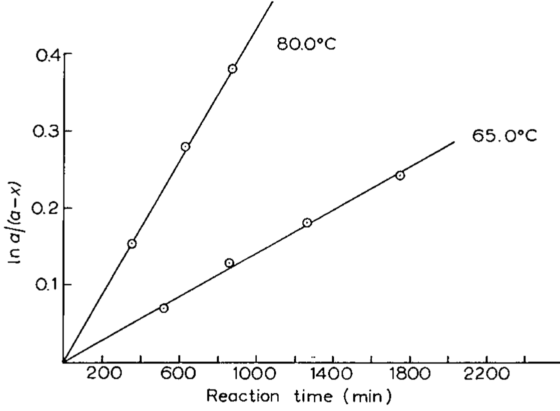

Fig. 3. Plot of ln $a/(a - x)$ *versus* time (min) for the TeMPm degradation to DITMP and MP.

TABLE III

Temperature 65.0°		Temperature 80.0°	
Time (min)	$k^1 \times 10^4$ (min^{-1})	Time (min)	$k^1 \times 10^3$ (min^{-1})
345	—*	355	1.00
516	3.43	635	1.02
856	3.52	879	1.00**
1,268	3.28		
1,748	3.20		

* k^1 not calculated, only trace DITMP formed.
** This result may be in error due to further degradation of ITMP.

From the data of Table III, it is proposed that:

$$\left.\begin{aligned} k^1_{65.0°} &= 3.37 \cdot 10^{-4}\ \text{min}^{-1} \\ k^1_{80.0°} &= 1.01 \cdot 10^{-3}\ \text{min}^{-1} \end{aligned}\right\} \text{at } 65.0°$$

and from this:

$$E^*_{\text{TeMPm}} \longrightarrow \text{DITMP} = 17.3\ \text{kcal/mole}$$

DISCUSSION

From these quantitative studies on the breakdown of the TeMPm anion to DITMP, and further to ITMP; the validity of the ring degradation is shown by two factors:

(i) The results obtained for k^1 on assuming ring degradation are consistent within themselves, and give a straight line when $\ln a/(a - x)$ is plotted against time, which passes through the origin.

(ii) The rate of appearance of phosphate species, recovered after 740 ml of eluant had passed through the column, was calculated to be identical (within experimental error) to that of the rate breakdown of DITMP under similar conditions (see ref. 5).

$$\left.\begin{aligned} k_{\text{DITMP} \to \text{ITMP}} &= 1.02 \cdot 10^{-3}\ \text{min}^{-1} \\ k_{\text{appearance}} &= 1.02 \cdot 10^{-3}\ \text{min}^{-1} \end{aligned}\right\} \text{at } 65.0°$$

Thus indicating that it was in fact ITMP being recovered from anion-exchange separation, the ITMP being resultant from the hydrolysis of DITMP.

The overall acid hydrolysis of the tetrametaphosphimate was a factor of some twenty-five times slower than that of its six-membered ring homologue trimetaphosphimate ($k^{65°}_{\text{TMPm}} = 8.9 \cdot 10^{-3}$ min^{-1}). The stability of TeMPm lies between that of DITMP and ITMP.

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SUMMARY

A kinetic study of the hydrolysis of ammonium tetrametaphosphimate in acid solution at elevated temperatures is described. The degradation of an eight-membered ring to a six-membered ring appears to be a general type reaction in such phosphorus–nitrogen systems.

REFERENCES

1 G. Pollard, G. Nickless and R. W. Warrender, *J. Chromatog.*, 9 (1962) 506.
2 A. Narath, F. H. Lohman and O. T. Quimby, *J. Am. Chem. Soc.*, 78 (1956) 4493.
3 A. Narath, F. H. Lohman and O. T. Quimby, *J. Am. Chem. Soc.*, 82 (1960) 1099.
4 F. H. Pollard, G. Nickless and R. W. Warrender, *J. Chromatog.*, 9 (1962) 493.
5 F. H. Pollard, G. Nickless and A. M. Bigwood, *J. Chromatog.*, 11 (1963) 534.
6 H. N. Stokes, *J. Am. Chem. Soc.*, 18 (1896) 780.
7 E. Steger and K. Lunkwitz, *Z. Anorg. Chem.*, 313 (1961) 262.
8 E. Steger and K. Lunkwitz, *Z. Anorg. Chem.*, 313 (1961) 271.

Notes

A gas chromatographic determination of pseudo-tropine in tropine

A mixture of tropine and pseudo-tropine, obtained, for instance, by reduction of tropinone, can be analysed fairly simply by the infrared spectrophotometric method of BECKETT *et al.*[1]. As, however, the absolute accuracy as stated by the authors is $\pm$ 3 %, this method is less reliable if the pseudoisomer content to be determined in the tropine sample is as low as 1 %.

The present paper reports a rapid gas chromatographic method for the analysis of mixtures of tropine and pseudo-tropine, with an absolute accuracy less than 0.5 %. While we were preparing the publication SEKERA AND SEKERA[2,3] published their findings of an electrophoretic method for the separation and estimation of these two isomers, which is also very accurate.

Apparatus

The analysis is performed with the aid of a Pye Argon Chromatograph provided with an ionisation detector with Ra-D source and a glass column of 1.20 m × 4 mm I.D.

The packing material used is Chromosorb P (175–210 μ), which is washed 3 times with half-saturated methanolic alkali, decanted and evaporated to dryness in a Rotavapor*.

The stationary phase, Apiezon L, is dissolved in chloroform and mixed with the support. The resulting mass is similarly evaporated to dryness in a Rotavapor, and allowed to dry overnight in a vacuum oven at 150°.

After the column has been filled, it is conditioned at 200° for several 24-h periods in a moderate argon flow in a separate oven. This oven was constructed in order to avoid unnecessarv operation of the gas chromatograph and to protect the detector from early soiling; it permits the conditioning of new columns as well as the storage of unused ones. Conditioning must be continued until resolution is adequate.

Results and discussion

Three different column packings were tried out, in an attempt to obtain complete separation of both isomers. The first trials with a column of 7 % (w/w) Apiezon L on Chromosorb P treated with methanolic alkali, were unsatisfactory since the peaks were highly asymmetrical ("tailing"). A column with the same percentage of stationary phase, on a support pre-treated with HCl and soda, as recommended by GOLDING *et al.*[4] for N-bases, did not produce better results. The tailing vanished completely on increasing the stationary phase to 15 % on Chromosorb P, prepared as indicated under Apparatus.

Any tropinone traces left the column first, followed by tropine and pseudo-tropine

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* Rotavapor: Firma Büchi, Flawil, Switzerland.

(Fig. 1). The KOVÁTS retention indices[5], found for this basic Apiezon column, are 1230, 1269 and 1340 respectively. The column, which has ± 1500 theoretical plates for tropine, has a high thermal stability; it has been in use for nearly two years.

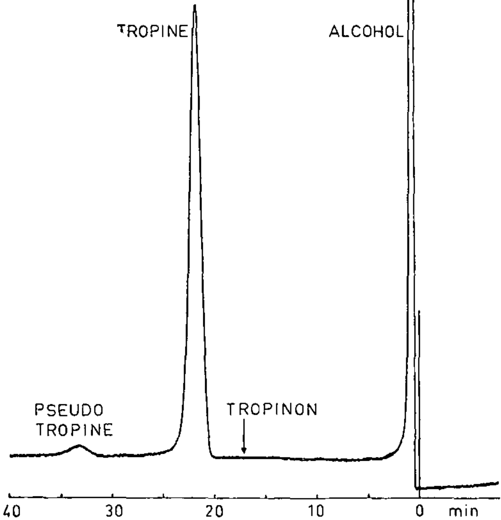

Fig. 1. Gas chromatogram of a tropine sample containing 5.6 % pseudo-tropine. Apparatus: Pye Argon Chromatograph with 1.20 m × 4 mm I.D. glass column. 15 % Apiezon L on basic Chromosorb P (175-210 μ). Temperature: 132°; gas velocity: 40 ml/min. Detector voltage: 1500 V. Sample: 0.2 μl of an alcoholic solution of tropine (10 mg/ml).

Quantitative determination

Among the usual methods for the quantitative interpretation of chromatograms the cut-and-weigh method is the only suitable one, if tropine samples with a pseudo content between 0 and 10 % are concerned.

The method advocated by PECSOK[6], however, which apparently has not been commonly accepted, also gives quick and accurate results[7,8]. It is based on the principle of the linear relation between the standard deviation σ of a normal distribution curve and the retention time t_R[9]. It is always more accurate to determine the latter value rather than the much lower σ value.

The area under a Gauss curve is given by:

$$A = 2.507\, h\, \sigma$$

where h is the peak height, so that with the relation $\sigma \sim t_R$ the product $t_R \times h$ represents the area formed by the curve. From the $t_R \times h$ values of both peaks the pseudo content may provisionally be determined from the formula

$$\text{percentage} = \frac{t_R \times h}{\Sigma(t_R \times h)} \times 100$$

The exact content is found from a calibration curve obtained by preparing a series of mixtures of the gas chromatographically pure components and plotting their $t_R \times h$

percentages *versus* the actual contents. Fig. 2 depicts such a curve, which holds for the column used under the conditions of temperature and gas rate mentioned. The S-shaped calibration curve intersects the 45°-line exactly at the point where isomer ratio and retention time ratio equal each other.

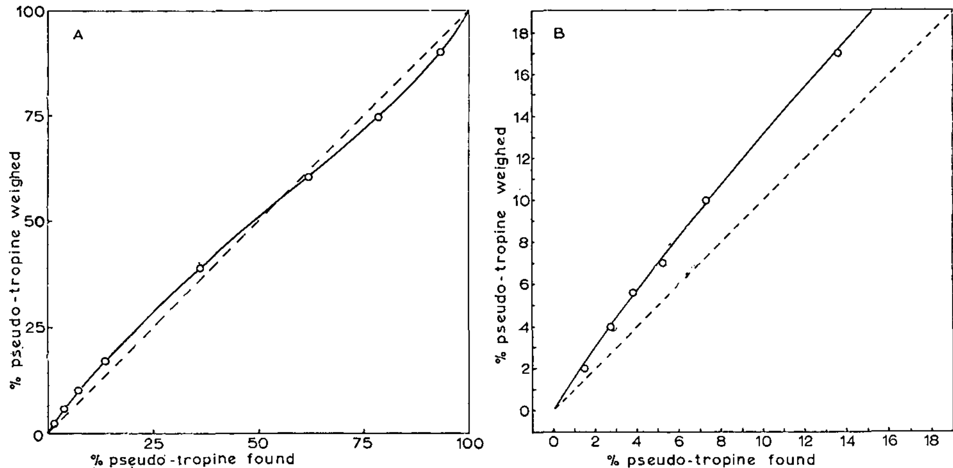

Fig. 2. True pseudo-tropine percentages *versus* those found by the present gas chromatographic method (Fig. 2 B is a detail enlargement of fig. 2 A).

Since measurements have proved that the standard deviations of the peaks for both components are independent of the amount of sample, the shape of the curve must probably be attributed to the non-linear nature of the detector response in this region (0.02–2 γ of substance). On re-evaluation of the curve only a small correction proved to be necessary after one year.

Analysis of mixtures of tropine and pseudo-tropine of known composition never revealed an absolute deviation exceeding 0.5 % of the pseudo content.

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1 A. H. Beckett, N. J. Harper, A. D. J. Balon and T. H. E. Watts, *Tetrahedron*, 6 (1959) 319.
2 A. Sekera and A. Sekera, *21e Congresso di Scienci Farmaceutiche Pisa*, 1961, p. 816-819.
3 A. Sekera, *Ann. Chim. (Paris)*, 7 (1962) 537.
4 W. E. Golding and A. C. Townsend, *Chem. Ind. (London)*, (1960) 1476.
5 E. Kováts, *Z. Anal. Chem.*, 181 (1961) 351.
6 R. L. Pecsok, *Principles and Practice of Gas Chromatography*, John Wiley and Sons, New York, 1959, p. 145.
7 K. K. Caroll, *Nature*, 191 (1961) 377.
8 H. Weiss and A. Kreyenbuhl, *Bull. Soc. Chim. France*, (1961) 603.
9 J. C. Bartlet and D. M. Smith, *Can. J. Chem.*, 38 (1960) 2057.

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Studies on a systematic analysis of terpenic hydrocarbons by gas–liquid chromatography

KLOUWEN AND TER HEIDE[1] have treated gas chromatographic data of over 22 isomeric terpenic hydrocarbons in connection with differences in polarisability. They concluded that for "non-polar" columns such as Apiezon-L or Silicone Oil a direct proportionality exists between the logarithm of retention volume and boiling point. Such columns were characterised as "non-selective" in contrast to the "selectivity" shown by polar chromatographic columns. The comparison of the logarithms of the retention volumes for one stationary phase with those in another stationary phase, first suggested by JAMES *et al.*[2], and used successfully in the identification and characterisation of members of homologous series, was found inapplicable to terpenic isomeric hydrocarbons. To obtain reasonable results, classification of terpenes into groups, based on rather vague structure similarities, was found necessary[1]. From other methods of coordinating gas chromatographic data, the method of WEHRLI AND KOVATS[3], where comparisons of "retention indices" are used, is applicable in homologous series but not always in the case of isomers.

Considering possibilities of developing graphical presentations for the identification of terpenic hydrocarbons from retention data given in the literature, we found that relative retention times, or volumes, or the logarithmic values, for one column, when plotted against those in another column of the "same selectivity", gave linear graphs. Since the term "same selectivity" is scarcely satisfied for columns of different stationary phases, relationships of data taken under different conditions but in columns of the same stationary phase were worked out. This linear relationship is still valid when flow rate, column dimensions, nature of carrier gas or temperature are varied (Figs. 1, 2 and 3). The results obtained can be correlated with those of JAMES *et al.*[2] in their relative retention volume logarithmic plots for two different stationary

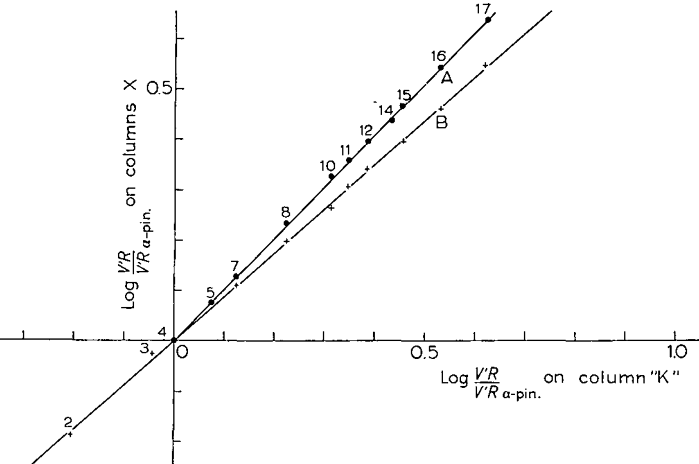

Fig. 1. Log (relative retention volumes) for "K" Perkin-Elmer column (Carbowax 1500 in Chromosorb W, length 4 m, helium flow 84 ml/min, temp. 100°) against those on Carbowax columns under different conditions. A[1] = 20% Carbowax 4000 on EMBACEL, length 3 m, hydrogen flow 75 ml/min, temp. 100°). B[5] = 28.5% Carbowax 4000 on Chromosorb C-44857, length 1.82 m, helium flow 45 ml/min, temp. 130°).

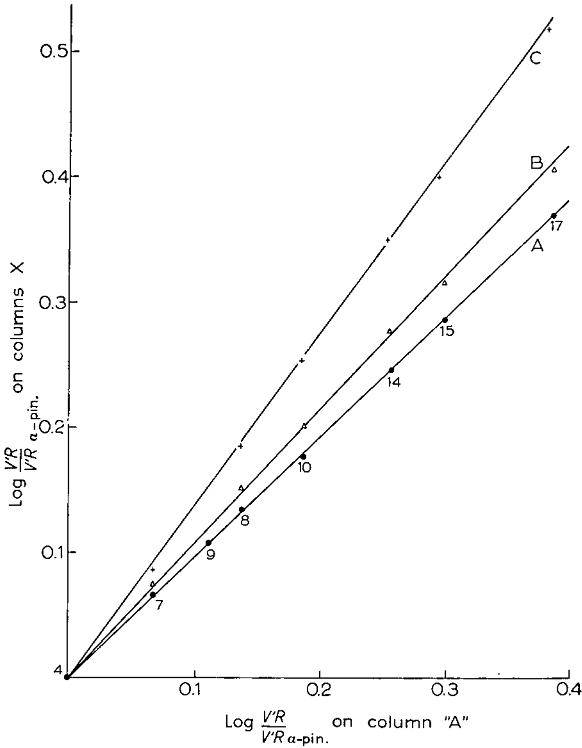

Fig. 2. Log (relative retention volumes) for "A" Perkin-Elmer column (diisodecyl phthalate, length 4 m, helium flow 86 ml/min, temp. 161°) against those on diisodecyl phthalate columns under different conditions. A = 34 % diisodecyl phthalate on Celite, length 4.5 m, helium flow 45 ml/min, temp. 150°. B = Perkin-Elmer "A" column, length 2 m, helium flow 50 ml/min, temp. 161°. C = Perkin-Elmer "A" column, length 2 m, helium flow 80 ml/min, temp. 99.5°.

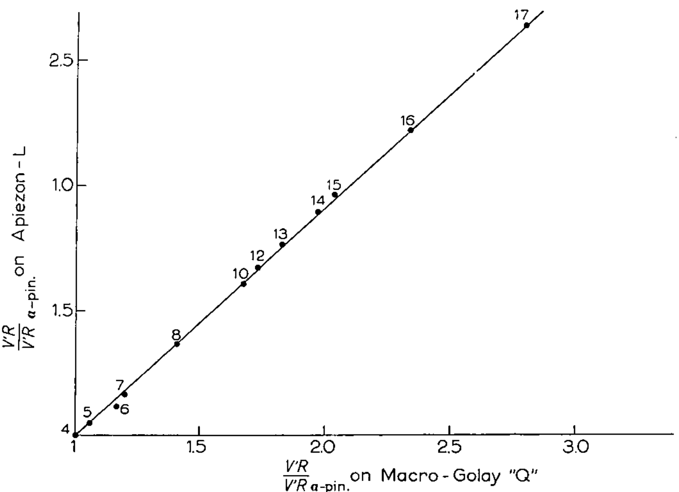

Fig. 3. Relative retention valumes for "Q" Perkin-Elmer Macro-Golay column (Apiezon L, length 100 m, helium flow 5 ml/min, temp. 133°) against those on a conventional column of the same stationary phase[1] (20 % Apiezon L, length 3 m, hydrogen flow 75 ml/min, temp. 120°).

phases, where the variation in column "selectivity" is replaced here by variation in structure.

No exceptions were observed in applying this graphical presentation. It was found, however, that small peak areas, when overlapping large peak areas, appear somewhat earlier. This phenomenon is related to the established fact that big size samples and highly concentrated sample components overload parts of the column, thus disturbing flow rate and column efficiency[4].

TABLE I

RETENTION VOLUMES OF TERPENE HYDROCARBONS RELATIVE TO THAT OF α-PINENE

	Hydrocarbon	"*K*"[a]	"*A*"[b]	"*Q*"[c]	"*R*"[d]
1	Bornylene	—	—	0.6	0.69
2	Cyclofenchene	0.623	0.685	0.77	0.8
3	Tricyclene	0.932	—	0.94	0.985
4	α-Pinene	1.00	1.00	1.00	1.00
5	β-Fenchene	1.18	1.12	1.05	1.12
6	α-Fenchene	1.30	—	1.12	1.23
7	Camphene	1.32	1.155	1.17	1.24
8	β-Pinene	1.67	1.365	1.37	1.5
9	Myrcene	2.04	1.285	—	—
10	3-Carene	2.05	1.535	1.61	1.81
11	α-Phellandrene	2.22	1.6	—	—
12	α-Terpinene	2.42	1.67	1.67	1.98
13	*p*-Cymene	—	—	1.76	2.61
14	Dipentene (limonene)	2.67	1.795	1.89	2.18
15	β-Phellandrene	2.86	1.95	1.96	—
16	γ-Terpinene	3.4	2.08	2.22	2.4
17	Terpinolene	4.16	2.465	2.64	3.14

[a] "K" = Perkin-Elmer column (Carbowax 1500 on Chromosorb W), length 4 m, helium flow 84 ml/min, temp. 100° ±0.5°.

[b] "A" = Perkin-Elmer column (diisodecyl phthalate on Chromosorb W), helium flow 86 ml/min, length 4 m, temp. 162° ±0.5°.

[c] "Q" = Perkin Elmer Macro-Golay column (Apiezon L), length 100 m, helium flow 5 ml/min, temp. 133° ±0.5°.

[d] "*R*" = Perkin-Elmer Macro-Golay column (polypropylene glycol UCON LB-550-X), helium flow 7 ml/min, length 100 m, temp. 132° ±0.5°.

The graphical presentation described is even more useful for identification purposes. Chemical reactions can lead to products whose nature is characteristic of the particular system. In examining the interaction of α-pinene with fatty acids we obtained in the chromatogram of the isomerisation products some 12 terpenic hydrocarbons for two of which we had no samples for comparison. By plotting our data successively against those of KLOUWEN AND TER HEIDE[1] and ZUBYK AND CONNER[5] we established that the unknown compounds coincide with β-fenchene and cyclofenchene. Later we succeeded in preparing pure samples of these two compounds and this result was further confirmed.

We have employed the Macro-Golay columns Apiezon-L and Polypropylene glycol (Perkin-Elmer Q and R columns) in the analysis or over 15 terpenic hydrocarbons with very satisfactory results[6]. These columns were found to cause some isomerisation during analysis, especially above 130°. The retention data obtained can be correlated with those obtained using conventional chromatographic columns (Fig. 3). Because of

the ideal equilibration conditions during analysis, these data are most reliable and could be suggested as standards.

Our results suggest that in order to compare gas chromatographic retention data, it is not necessary to reproduce working conditions. A chromatogram obtained under a definite set of conditions can be compared with one obtained under different conditions, providing equilibrium of flow rate and temperature is attained and columns of the same stationary phase are used. The validity of this statement has been proved for the terpenic hydrocarbons; we do not see why this should not apply to other systems.

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1 M. H. KLOUWEN AND R. TER HEIDE, *J. Chromatog.*, 7 (1962) 297.
2 A. T. JAMES, A. J. P. MARTIN AND G. H. SMITH, *Biochem. J.*, 52 (1952) 238.
3 A. WEHRLI AND E. KOVATS, *Helv. Chim. Acta*, 42 (1959) 2728.
4 S. DAL NOGARE AND R. S. JUVET, JR., *Gas-Liquid Chromatography*, Interscience, New York–London, 1962, p. 16.
5 W. J. ZUBYK AND A. Z. CONNER, *Anal. Chem.*, 32 (1960) 912.
6 G. VALKANAS AND N. ICONOMOU, *Pharm. Acta Helv.*, in the press.

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Gas chromatographic analysis of polar–non polar mixtures

In the study of thermodynamic properties of multicomponent mixtures, such as vapor–liquid equilibrium compositions, the gas chromatographic technique has been used extensively. The methods commonly used in the determination of concentrations from a chromatograph assume that either the peak height or the peak area is proportional to the concentrations of the components present in the mixture. Recently, WAGNER AND WEBER[1] studied polar–non polar mixtures, such as ethanol–benzene–heptane, and proposed a modified method in which the mole fraction ratio to the peak area ratio is considered constant, namely:

$$\frac{(x_i/x_j)_{\text{mole fraction}}}{(x_i/x_j)_{\text{peak area}}} = K_{ij} \qquad (1)$$

These constants and the condition that the sum of the mole fractions must be equal to unity were used to calculate the compositions.

We have determined the composition of ethanol–benzene–cyclohexane mixtures in this laboratory, using a Perkin-Elmer Model 154C Vapor Fractometer coupled with a Leeds and Northrup series 6000 Recorder and a Perkin-Elmer Model 194 Integrator. In the Vapor Fractometer, two 2-m type R columns were employed in series to separate the three components. A curve was obtained on a $(x_i/x_j)_{\text{mole fraction}}$ *vs.* $(x_i/x_j)_{\text{peak area}}$

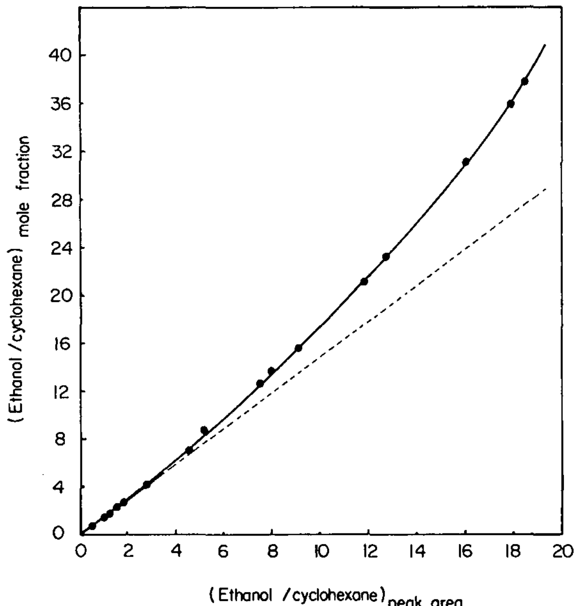

Fig. 1. Relationship between peak area ratios and mole fraction ratios of ethanol–cyclohexane in the ethanol–benzene–cyclohexane mixture.

plot for the components ethanol and cyclohexane in the ternary mixture as shown in Fig. 1; eqn. (1) was only valid over a limited concentration range. This non-linear behavior presented difficulties in extrapolation. However, when $(x_i/x_j)_{\text{mole fraction}}/(x_i/x_j)_{\text{peak area}}$ was plotted against $(x_i/x_j)_{\text{peak area}}$, a straight line was obtained as shown in Fig. 2. The value of the constant b of the linear relationship:

$$\frac{(x_i/x_j)\ \text{mole fraction}}{(x_i/x_j)\ \text{peak area}} = a + b\left(\frac{x_i}{x_j}\right)_{\text{peak area}} \tag{2}$$

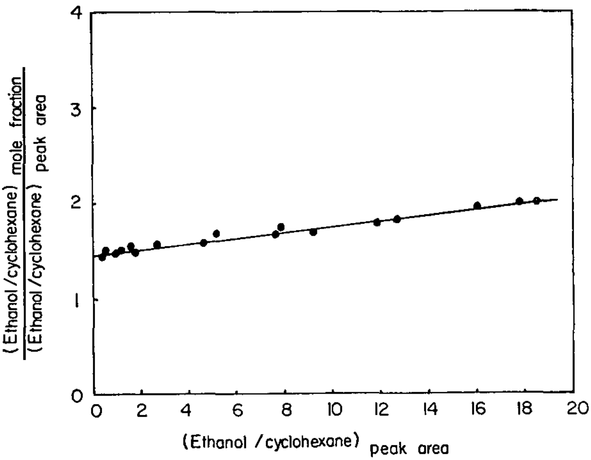

Fig. 2. Replot of data in Fig. 1 according to eqn. (2).

is small, indicating that at low $(x_i/x_j)_{\text{peak area}}$ values, eqn. (2) can be reduced to eqn. (1). However, at high $(x_i/x_j)_{\text{peak area}}$ values, considerable error would be introduced if the term $b(x_i/x_j)_{\text{peak area}}$ were neglected.

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[1] I. F. WAGNER AND J. H. WEBER, *Ind. Eng. Chem., Chem. Eng. Data Ser.*, 3 (1958) 220.

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Revelation von Carbobenzoxy-Aminosäuren auf Dünnschicht-chromatogrammen

Zur Revelation von Carbobenzoxy(Cbo)-Aminosäuren verwenden EHRHARDT UND CRAMER[1] $K_2Cr_2O_7$ in konz. H_2SO_4. Die Erfassungsgrenze liegt bei etwa 3 μg.

Wir haben gefunden, dass die modifizierte Chlor–Tolidin-Reaktion[2] wesentlich sensitiver ist. Bei allen untersuchten Cbo-Aminosäuren* liessen sich Mengen von 0.5 μg noch deutlich erkennen.

Ausführung. Die Platte mit den zu revelierenden Substanzen wird, zwecks Befeuchtung der Schicht, kurz über kochendes Wasser gehalten und anschliessend mit Chlor behandelt[2]. Vor dem Besprühen lässt man die Schicht 2–3 min gut lüften.

Sprühreagens[2]: 80 mg *o*-Tolidin + 15 ml Eisessig + 0.5 g Jodkali werden mit dest. Wasser auf 250 ml aufgefüllt.

Man besprüht zunächst eine Ecke des Chromatogramms; wird der Untergrund blau so muss die Platte länger gelüftet werden.

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[1] E. ERHARDT UND F. CRAMER, *J. Chromatog.*, 7 (1962) 405.
[2] M. BRENNER, A. NIEDERWIESER UND G. PATAKI, in E. STAHL, *Dünnschichtchromatographie*, Springer Verlag, Berlin–Göttingen–Heidelberg, 1962, S. 425.

Eingegangen den 10. April 1963

* Zwanzig Cbo-Aminosäuren in drei Fliessmitteln.
** Direktor Prof. Dr. TH. KOLLER.

Procédé de chromato-électrophorèse permettant d'obtenir la séparation des dinitrophényl-aminoacides hydrosolubles

Si les procédés chromatographiques[1,2] d'identification des DNP-aminoacides* ethéro-solubles sont parfaitement au point, il n'en est pas de même de ceux qui peuvent s'appliquer à l'analyse des DNP-aminoacides hydrosolubles. Ainsi la caractérisation de ces derniers était, jusqu'ici, une opération analytique délicate malgré les nombreuses méthodes de chromatographie proposées**. Cette analyse est souvent rendue difficile par suite de la présence d'un grand excès de l'un des DNP-dérivés (ε-DNP-lysine) et de nombreux artéfacts colorés. Le seul procédé d'analyse satisfaisant était l'électrophorèse sur papier à une dimension dans l'ammoniaque N[3]. Au cours de l'emploi de ce procédé, nous avons été amené à essayer un certain nombre d'amines volatiles ou d'électrolytes alcalins non volatils qui peuvent être avantageusement utilisés à la place de l'ammoniaque. Nous nous proposons de donner ici les positions des DNP-aminoacides hydrosolubles et de quelques artéfacts dans un système de chromato-électrophorèse.

Dans les diagrammes de la Fig. 1, donnant les positions des taches de DNP-aminoacides, la 1-ère dimension (feuille, 39 × 46 cm, de papier Whatman No. 20) du

TABLEAU I

DISTANCES (Δ) ENTRE LES TACHES D' α-MONO-DNP-ARGININE ET D'ε-MONO-DNP-LYSINE APRÉS ÉLECTROPHORÈSE DANS DIVERS ÉLECTROLYTES ALCALINS[a]

	Électrolytes	*Distance Δ (cm)*	*Temps*	*Volts*	*mA*
a	Borax 0.025 *M* (pH 8.92)	1.4	4h 20	425	12.2
b	Diéthylamine 0.025 *M*	4.7	4h 25	442	9.2
c	Triéthylamine 0.25 *M*	4.5	4h 20	442	8.8
d	Ammoniaque 0.25 *M*	3.2	4h 20	430	11.2
e	Veronal sodé à 0.5 % (pH 9.8)	0.7	4h 30	428	9.2
f	Phosphate trisodique 0.0025 *M*	1.4	4h 30	448	5.8
g	Acide acétique (0.2 *M*), Ammoniaque (pH 7.55)	0.3	4h 10	432	9.6
h	Bicarbonate d'ammonium 0.02 *M*	1.1	3h 31	432	9.2
i	Diéthylamine 0.05 *M*	4.3	4h 20	445	11.5

[a] Électrophorèses réalisées dans un appareil en toit type DURRUM[4] pour feuille, 39 (largeur) × 46 cm, de papier Whatman No. 20; ligne de répartition des substances à 11 cm de l'extrémité "catodique" de la feuille; générateur de tension muni d'une valve 5 Z 3 (350 × 350 Veff), filtre en π; résistance de 100,000 Ω branchée en parallèle avec la cellule d'électrophorèse à la sortie du générateur.

chromato-électrogramme (pré-équilibré pendant 4 h dans une cuve de 105 l avec 500 ml d'ammoniaque 0.8 N) a été développée (24 h), avec écoulement continu du solvant[1] toluène–chloro-2 éthanol–pyridine–0.8 N ammoniaque (150:90:45:90) (préparé 4 h avant emploi et filtré). Ainsi la tache de dinitroaniline est sortie du chromatogramme,

* Abréviations: DNP = radical 2,4-dinitrophényl; α-Arg = α-mono-DNP-arginine; CySCM = DNP-S-carboxyméthylcystéine ("marqueur"); $CySO_3H$ = acide DNP-cystéique; DNPOH = dinitrophénol ("marqueur"); α-His = α-mono-DNP-histidine; di-His = di-DNP-histidine; H_1 = trace de produit non identifié; α-Lys = α-mono-DNP-lysine; ε-Lys = ε-mono-DNP-lysine; α-Orn = α-mono-DNP-ornithine; δ-Orn = δ-mono-DNP-ornithine; S_1, S_2, S_3 = artéfacts issus de la S-mono-DNP-cystéine; Taur = DNP-taurine.

** Pour une revue voir réf. 3.

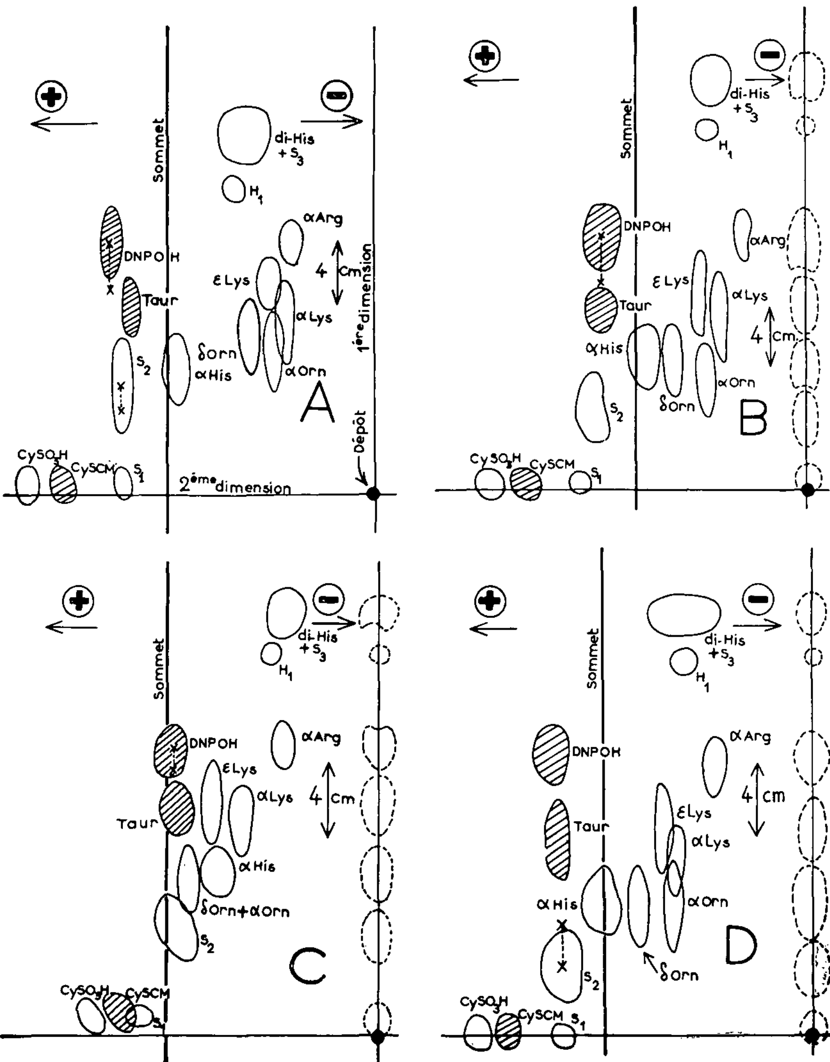

Fig. 1. Diagrammes donnant les positions des taches des DNP-aminoacides hydrosolubles et de quelques artéfacts, après chromato-électrophorèse. 1-ère dimension: chromatographie (conditions opératoires indiquées dans le texte); 2-ème dimension: électrophorèse (durée: 4 h 20 min; conditions opératoires indiquées dans le Tableau I) avec les électrolytes du Tableau I: a en A, b en B, c en C, d en D; en traits interrompus: positions des substances après chromatographie; taches hachurées: position de "marqueurs" facilitant les identifications.

celle de la di-DNP-histidine se situe à 4–6 cm de l'extrémité de la feuille et celles des DNP-aminoacides se trouvent réparties en 5–6 groupes sur une ligne placée à 11 cm du côté catodique. Le chromatogramme sec est disposé pour l'électrophorèse, en seconde dimension, dans les électrolytes dont la composition est indiquée dans le Tableau I.

Comme on peut le voir dans le Tableau I et dans les diagrammes de la Fig. 1, le meilleur système est celui qui utilise la diéthylamine 0.025 *M* comme électrolyte pour

l'électrophorèse en 2-ème dimension. Le seul inconvénient de ce procédé est la difficulté de mettre en évidence les aminoacides non substitués, par la réaction à la ninhydrine, à cause de la présence de substances donnant une réaction positive à la ninhydrine dans les amines volatiles utilisées et de l'existence d'une large bande d'éthanolamine formée au cours du développement chromatographique en 1-ère dimension*. Sur les diagrammes de la Fig. 1 ont été indiquées les positions des artéfacts (S_1, S_2, S_3) issus de la décomposition de la S-mono-DNP-cystéine**. Les deux produits dérivés principaux S_2 et S_3 sont indistingables, par ce procédé d'analyse, respectivement de la N,N'-di-DNP-cystine (pour S_2) et de la N,S-di-DNP-cystéine (pour S_3).

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1 G. BISERTE ET R. OSTEUX, *Bull. Soc. Chim. Biol.*, 33 (1951) 50.
2 A. L. LEVY, *Nature*, 174 (1954) 126.
3 G. BISERTE, J. W. HOLLEMAN, J. HOLLEMAN-DEHOVE ET P. SAUTIÈRE, *Chromatographic Reviews* Vol. 2, Elsevier, Amsterdam, 1960, p. 59.
4 E. L. DURRUM, *J. Am. Chem. Soc.*, 72 (1950) 2943.
5 H. ZAHN ET K. TRAUMANN, *Z. Naturforsch.*, 9b (1954) 518.

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* Cette mise en évidence est facile sur les chromato-électrogrammes réalisés avec le système: *n*-butanol–diéthylaminoéthanol–eau (80:5:15) puis diéthylaminoéthanol 0.025 *M*.

** Produit préparé à pH 5.2 selon réf. 5 et donnant une seule tache, très distincte de celle de la N,N'-di-DNP-cystine et de la N,S-di-DNP-cystéine, par chromatographie dans le système *n*-butanol–acide acétique–eau (70:7:23) puis phénol saturé d'eau; notons que la S-mono-DNP-cystéine est une substance peu stable.

An effect of the sample pH on the separation of phosphates by ion-exchange chromatography*

Ion-exchange separation of the acid soluble organic phosphates of protein-free trichloroacetic acid extracts of milk[1] showed in preliminary studies that inorganic orthophosphate was eluted as two peaks if the pH of the extract was 4. If the pH of the extract was adjusted with ammonia to 6.4, the first peak decreased in area while the second increased.

These observations indicated the elution of inorganic phosphate from the ion-exchange resin was dependent on the pH of the sample put on the column, and that orthophosphate could, under certain circumstances, behave as two distinct compounds. Since the effect to our knowledge has not been the subject of previous investigation, this note appeared to be worthwhile.

Potassium dihydrogen phosphate (41 mg) containing 10 mg of phosphorus was dissolved in 10 ml of water. The solution (pH 4.7) was charged to a 25 × 1 cm Dowex

* From the Ph. D. thesis of the author, Massachusetts Institute of Technology. Contribution No. 588 from the Department of Nutrition and Food Science.

1 — X8 column in the chloride form, followed by 100 ml of water. Concave gradient elution (using a two-reservoir system with the second reservoir in the shape of a cone) with 0–1 *M* NH_4Cl was carried out. The effluent from the column was collected in 10 ml fractions and was analyzed for the presence of inorganic phosphate according to BARTLETT[2].

A solution (110 ml, pH 8.0) of disodium hydrogen phosphate containing 10 mg of phosphorus was chromatographed the same way. The two elution diagrams (Fig. 1 and Fig. 2) illustrate the effect of the pH of the sample solution. At pH 4.7 two peaks

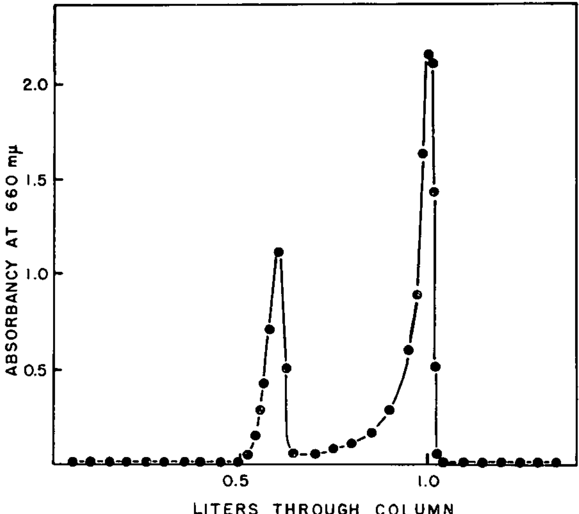

Fig. 1. Elution of inorganic phosphate. pH of sample = 4.7.

were obtained, while only one appeared at pH 8.0. The last peak in the first diagram occupied a position identical to the single peak in the second diagram.

The results showed that the ionic form in which inorganic phosphate was initially absorbed by the column was to some degree determined by the pH of the sample solution.

At pH 8.0 the phosphate is predominately present as monohydrogen phosphate ions, while pH 4.7 favors the dihydrogen phosphate ion. The relative amounts of divalent *vs.* monovalent ions absorbed by the resin will also be influenced by the rate constant of the exchange reactions:

$$2\ RCl + HPO_4^{-} \leftrightharpoons R_2HPO_4 + 2\ Cl^{-} \quad (1)$$

$$RCl + H_2PO_4^{-} \leftrightharpoons RH_2PO_4 + Cl^{-} \quad (2)$$

where R designates the cationic groups of the resin. The results indicate that at pH 8.0 the conditions are such that virtually all of the orthophosphate is absorbed as divalent ions.

It is reasonable to believe that this effect of pH of the sample solution on the valency of ions absorbed on the resin is not limited to orthophosphate but also may

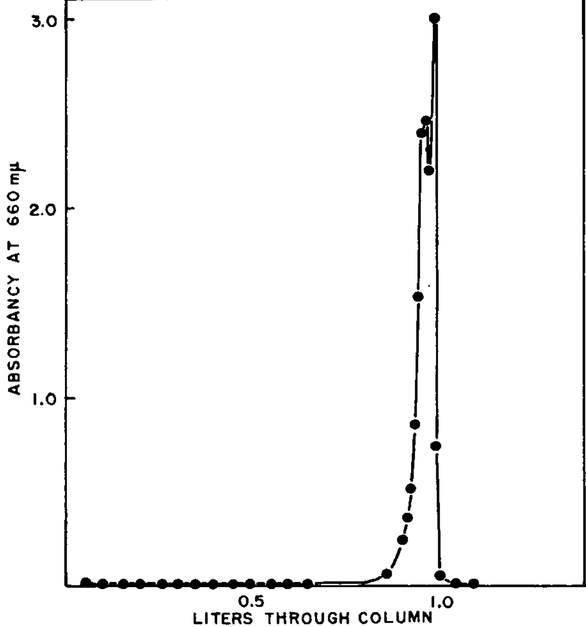

Fig. 2. Elution of inorganic phosphate. pH of sample = 8.0.

occur with organic phosphates, nucleotides, etc. Thus the possibility exists that artifactual peaks may occur in ion-exchange chromatography of polyvalent ions unless the pH of the sample is properly adjusted.

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1 J. E. HOFF AND E. L. WICK, Acid-soluble Organic Phosphates in Cow's Milk, *J. Food Sci.*, (in the press).
2 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.

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TABLE 1

R_F VALUES OF SOME AMINO ACID ESTERS AND RELATED COMPOUNDS

(Z. A. SHABAROVA, N. A. HUGHES AND J. BADDILEY, *Biochem. J.*, 83 (1962) 216)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:1, by vol.).
S_2 = Butan-1-ol–acetic acid–water (5:2:3, by vol.).
S_3 = Propan-1-ol–NH_4OH (sp. gr. 0.88)–water (6:3:1, by vol.).
Paper: Whatman No. 1 (ascending).
Detection: D_1 = Ninhydrin reagent (R. CONSDEN AND A. H. GORDON, *Nature*, 162 (1948) 180) for amino acids.
D_2 = Sodium periodate–Schiff reagents (J. G. BUCHANAN, C. A. DEKKER AND A. G. LONG, *J. Chem. Soc.*, (1950) 3162; J. BADDILEY, J. G. BUCHANAN, R. E. HANDSCHUMACHER AND J. F. PRESCOTT, *J. Chem. Soc.*, (1956) 2818), for ethylene glycol.
D_3 = Perchloric acid–ammonium molybdate reagent (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107), for phosphates.
D_4 = $FeCl_3$ reagent (E. R. STADTMAN AND H. A. BARKER, *J. Biol. Chem.*, 184 (1950) 769), for hydroxamates.

Compound	S_1	S_2	S_3
N-Benzyloxycarbonylalanine hydroxamate	0.95	—	—
Alanine hydroxamate	0.35	—	—
Alanine amide	—	—	0.59
Alanine	—	0.42	0.48
2-DL-Alanyloxyethanol	—	0.52	—
2-DL-Alanyloxyethyl dihydrogen phosphate	—	0.33	—
2-Hydroxyethyl dihydrogen phosphate	—	0.34	0.24
Ethylene-1,2-phosphate	—	0.31	0.24

TABLE 2

ELECTROPHORETIC MOBILITIES OF SOME AMINO ACID ESTERS AND RELATED COMPOUNDS

(Z. A. SHABAROVA, N. A. HUGHES AND J. BADDILEY, *Biochem. J.*, 83 (1962) 216)

Electrolyte: 0.1 *M* sodium acetate (pH 6.0).
Paper: Whatman No. 1.
Potential gradient: 10 V/cm.
Time of run: 2 h.
Mobility: M (cm towards cathode).
Detection: D_1 = Ninhydrin reagent (R. CONSDEN AND A. H. GORDON, *Nature*, 162 (1948) 180), for amino acids.
D_2 = Sodium periodate–Schiff reagents (J. G. BUCHANAN, C. A. DEKKER AND A. G. LONG, *J. Chem. Soc.*, (1950) 3162; J. BADDILEY, J. G. BUCHANAN, R. E. HANDSCHUMACHER AND J. F. PRESCOTT, *J. Chem. Soc.*, (1956) 2818), for α-glycols.
D_3 = Perchloric acid–ammonium molybdate reagent (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107), for phosphates.

Compound	*M*
Alanine	+ 2.3
2-DL-Alanyloxyethanol	+ 8.3
2-DL-Alanyloxyethyl dihydrogen phosphate	+ 1.0
2-Hydroxyethyl dihydrogen phosphate	— 4.7

TABLE 3

R_F VALUES OF ETHYLMERCAPTURIC ACID AND S-ETHYL-L-CYSTEINE

(A. E. R. THOMSON, E. A. BARNSLEY AND L. YOUNG, *Biochem. J.*, 86 (1963) 145)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5, by vol.).
S_2 = Molten phenol–water (4:1, v/v).
S_3 = Lutidine–collidine–water (6:5:5, by vol.).
S_4 = Butan-1-ol–pyridine–3 *N* NH_3 soln. (4:3:3, by vol.).
S_5 = 2-Methylbutan-2-ol–pyridine–water (7:7:6, by vol.).
Paper: Whatman No. 1 (ascending or descending; not more closely specified).
Detection: D_1 = $K_2Cr_2O_7$–$AgNO_3$ spray reagent (R. H. KNIGHT AND L. YOUNG, *Biochem. J.*, 70 (1958) 111) and dipping in Pt reagent (G. TOENNIES AND J. J. KOLB, *Anal. Chem.*, 23 (1951) 823), for both S-ethyl-L-cysteine and ethylmercapturic acid.
D_2 = Ninhydrin reagent (G. TOENNIES AND J. J. KOLB, *loc. cit.*), for S-ethyl-L-cysteine.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Ethylmercapturic acid	0.78, 0.85, 0.87	0.82, 0.78, 0.83	0.50, 0.55	0.56	
S-Ethyl-L-cysteine	0.44, 0.45, 0.53	0.73, 0.76, 0.77		0.42, 0.46	0.55

TABLE 4

R_F VALUES OF L-THREONINE ETHANOLAMINE PHOSPHATE AND CLOSELY RELATED COMPOUNDS

(H. ROSENBERG, A. H. ENNOR, D. D. HAGERMAN AND S. SUGAI, *Biochem. J.*, 84 (1962) 536)

Solvents: S_1 = Ethanol–formic acid (98–100 %, w/v; British Drug Houses Ltd.)–water (7:1:2, by vol.).
S_2 = Water-saturated phenol.
Paper: Whatman No. 3 (ascending).
Detection: D_1 = 0.2 % (w/v) ninhydrin in acetone (papers air-dried at 30°, dipped, then heated (10 min, 80°).
D_2 = Orthophosphate method (H. ROSENBERG, *J. Chromatog.*, 2 (1959) 487).

Compound	R_F	
	S_1	S_2
L-Threonine ethanolamine phosphate (2-aminoethyl L-2-amino-2-carboxy-1-methyl-ethyl hydrogen phosphate)	0.29	0.38
Serine ethanolamine phosphate	0.24	0.24

TABLE 5

R_F VALUES OF FOUR PHOSPHOLIPIDS

(R. COLLIER, *Nature*, 194 (1962) 771)

Solvent: Di-*n*-butyl ether–propionic acid (2:1, v/v) saturated with zinc chloride.
Paper: Whatman No. 1 (ascending).
Impregnation: Paper soaked for 5 min in 3.5% HCl saturated with zinc oxide (at 60°, solution being cooled and filtered befor use), drained, blotted, allowed to lie 2–3 sec under stream of hot air. Phospholipids may be applied before or after impregnation).
Length of run: 27 cm.
Time of run: 4 h.
Detection: "Solochrome" technique applied (after paper had been dried in a stream of hot air).

Compound	R_F
Phosphatidyl ethanolamine	0.32–0.40
Lecithin	0.20–0.25
Lysophosphatidyl ethanolamine	0.13–0.18
Lysolecithin	0.05–0.08

TABLE 6

R_F AND RELATIVE R_F VALUES OF MONOSACCHARIDES AND THEIR MONO- AND DI-SULPHATES

(A. G. LLOYD, *Biochem. J.*, 83 (1962) 455)

Solvents: S_1 = Butan-1-ol–acetic acid–water (50:12:25, by vol.).
S_2 = Butan-1-ol–ethanol–water (3:1:1, by vol.) containing 3% (w/v) cetyl pyridinium chloride (D. A. REES, *Nature*, 185 (1960) 309).
Paper: Whatman No. 1 (descending).
Temperature of run: 20°.
Time of run: 48 h (S_1); 20 h (S_2).
Detection: D_1 = Alkaline $AgNO_3$ reagent (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 174 (1950) 444).
D_2 = *p*-Anisidine reagent (L. HOUGH, J. K. N. JONES AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702).
D_3 = Hexosamine reagent (S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238).
D_4 = Sulphate ester reagent (A. G. LLOYD, *Biochem. J.*, 75 (1960) 478).

Original compound	R_{Glu}* (S_1)			R_F (S_2)
	Monosaccharide	*Monosulphate*	*Disulphate*	*6-O-Sulphate*
D-Glucose	1.0	0.66	0.42	0.44
D-Galactose	0.92	0.58	0.43	0.40
N-Acetyl-D-glucosamine	1.24	0.76	0.54	—
N-Acetyl-D-galactosamine	1.13	0.72	0.54	—
D-Fucose	1.5	0.92	0.59	—
L-Fucose	1.47	0.98	0.59	—

* $R_{Glu} = R_F$ compound/R_F glucose.

TABLE 7

R_F AND RELATIVE R_F VALUES OF TEICHOIC ACID DEGRADATION PRODUCTS

(J. BADDILEY, J. G. BUCHANAN, U. L. RAJBHANDARY AND A. R. SANDERSON, *Biochem. J.*, 82 (1962) 439)

Solvents: S_1 = Propan-1-ol–aq. NH_3 soln. (sp. gr. 0.88)–water (6:3:1, v/v) (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107).
S_2 = Propan-2-ol–conc. HCl–water (65:17:18, v/v) (J. D. SMITH AND G. R. WYATT, *Biochem. J.*, 49 (1951) 144).
S_3 = Butan-1-ol–ethanol–water– aq. NH_3 soln. (sp. gr. 0.88) (40:10:49:1, v/v; organic phase) (A. B. FOSTER, D. HORTON AND M. STACEY, *J. Chem. Soc.*, (1957) 81).
S_4 = Butan-1-ol–pyridine–water (6:4:3, v/v) (A. JEANES, C. S. WISE AND R. J. DIMLER, *Anal. Chem.*, 23 (1951) 415).
S_5 = Pyridine–ethyl acetate–water–acetic acid (5:5:3:1, v/v) (F. G. FISCHER AND H. G. NEBEL, *Z. Physiol. Chem.*, 302 (1955) 10).

Paper: P_1 = Whatman No. 4 (ascending).
P_2 = Whatman No. 4 (descending).
P_3 = Whatman No. 1 (descending).

Detection: D_1 = Periodate–Schiff reagent (J. BADDILEY, J. G. BUCHANAN, R. E. HANDSCHUMACHER AND J. F. PRESCOTT, *J. Chem. Soc.*, (1956) 2818), for α-glycols.
D_2 = Ninhydrin reagent (R. CONSDEN AND A. H. GORDON, *Nature*, 162 (1948) 180), for amino acids and amino sugars.
D_3 = Molybdate reagent (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107) for phosphate esters.
D_4 = Aniline phthalate reagent (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444), for sugars.

Compound	R_F		R_{Rib}*		R_{Glu}*
	S_1P_1	S_2P_1	S_3P_2	S_4P_3	S_5P_3
Ribitol	0.63	0.68	1.00	1.00	1.58
Glycerol	0.72	0.80	1.53	1.50	—
Anhydroribitol	0.72	0.80	1.42	1.43	—
Glucosamine	0.63	0.45	0.78	0.61	1.00
N-Acetylglucosamine	0.67	—	1.05	1.12	1.68
Alanine	0.60	0.65	—	—	—
Glucosaminylribitol	0.54	0.24	0.47	0.53	0.83
N-Acetylglucosaminylribitol	0.54	0.62	0.47	0.68	1.26
Glucosaminylribitol phosphates	0.25	—	—	—	—
2,5-Anhydromannose	0.65	—	—	1.40	—
α-Methyl-N-acetylglucosaminide	—	—	1.70	—	—
β-Methyl-N-acetylglucosaminide	—	—	1.50	—	—

* R_{Rib} = R_F compound/R_F ribitol; R_{Glu} = R_F compound/R_F glucosamine.

TABLE 8

R_F AND RELATIVE R_F VALUES OF TEICHOIC ACID DEGRADATION PRODUCTS

(J. BADDILEY, J. G. BUCHANAN, R. O. MARTIN AND U. L. RAJBHANDARY, *Biochem. J.*, 85 (1962) 49)

Solvents: S_1 = Propan-1-ol-aq.NH_3 soln. (sp. gr. 0.88)–water (6:3:1) (C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107).
S_2 = Propan-2-ol-aq. NH_3 soln. (sp. gr. 0.88)–water (7:1:2).
S_3 = Butan-1-ol–ethanol–water–aq. NH_3 soln. (sp. gr. 0.88) (40:10:49:1; organic phase) (A. B. FOSTER, D. HORTON AND M. STACEY, *J. Chem. Soc.*, (1957) 81).
Paper: Whatman No. 4, previously washed with 2 *N* acetic acid, then water; ascending (S_1), descending (S_2, S_3).
Detection: D_1 = Periodate–Schiff reagent (J. BADDILEY, J. G. BUCHANAN, R. E. HANDSCHUMACHER AND J. F. PRESCOTT, *J. Chem. Soc.*, (1956) 2818), for α-glycols.
D_2 = Molybdate reagent (C.S. HANES AND F. A. ISHERWOOD, *loc. cit.*), for phosphates.
D_3 = Alkaline $AgNO_3$ reagent (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444), for reducing sugars.
D_4 = Ninhydrin (R. CONSDEN AND A. H. GORDON, *Nature*, 162 (1948) 180), for amino acids and amino sugars.

Compound	R_F	$R_{Rib\text{-}P}$*	R_{Rib}**
	S_1	S_2	S_3
Ribitol	0.64	—	1.00
Anhydroribitol	0.74	—	1.39
Glucosamine	0.62	—	0.73
2,5-Anhydromannose	0.65	—	—
Ribitol-1-phosphate	0.25	1.00	—
Ribitol-2-phosphate	0.29	1.06	—
Glucosaminylribitol-1-phosphate	0.25	0.86	—
Glucosaminylribitol-2-phosphate	0.25	0.92	—
Glucosaminylribitol diphosphates } Ribitol diphosphate }	0.08–0.15	—	—

* $R_{Rib\text{-}P}$ = R_F compound/R_F ribitol-1-phosphate.
** R_{Rib} = R_F compound/R_F ribitol.

TABLE 9

ELECTROPHORETIC MOBILITIES OF GLUCOSAMINYL- AND N-ACETYL-GLUCOSAMINYL-RIBITOL PHOSPHATES

(J. BADDILEY, J. G. BUCHANAN, U. L. RAJBHANDARY AND A. R. SANDERSON, *Biochem. J.*, 82 (1962) 439)

Electrolyte: 0.1 *M* ammonium acetate buffer, pH 3.8.
Paper: Whatman No. 1.
Potential applied: 4 V/cm.
Time of run: 7 h.
Mobility: M = cm towards anode.
Detection: Not specified.

Compound	*M*
Glucosaminylribitol phosphates	3.5
N-Acetylglucosaminylribitol phosphates	11.5

TABLE 10

R_F VALUES OF TWO THIADIAZOLYL-HYDRAZONES

(K. ARVIDSSON AND J. SANDSTRÖM, *Acta Chem. Scand.*, 15 (1961) 1179)

Solvent: Benzene (see B. WIKBERG, *Acta Chem. Scand.*, 12 (1958) 615).
Paper: Whatman No. 1 (*cf.* B. WIKBERG, *loc. cit.*).
Impregnation: Dimethylsulphoxide (*cf.* B. WIKBERG, *loc. cit.*).
Detection: U.V. light.

Compound	R_F*
Cyclohexanone 4-methyl-1,3,4-thiadiazole-5(4)-thion-2-yl-hydrazone	0.33
Cyclohexanone 5-methylthio-1,3,4-thiadiazol-2-yl-hydrazone	0.45

* Approximate.

TABLE 11

R_F VALUES (THIN LAYER) OF 16α,17-EPOXY-Δ^5-PREGNEN-3β-OL-20-ONE AND RELATED STEROIDS

(V. SCHWARZ, *Collection Czech. Chem. Commun.*, 27 (1962) 2567)

Solvents: S_1 = Petroleum ether.
S_2 = Petroleum ether–benzene (2:1).
S_3 = Petroleum ether–benzene (1:1).
S_4 = Benzene.
S_5 = Benzene–ethanol (95:5).
Thin-layer carrier: Acid Al_2O_3 (acetic acid deactivated); for other details *cf.* S. HEŘMÁNEK, V. SCHWARZ AND Z. ČEKAN, *Pharmazie*, 16 (1961) 566.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
16α,17-Epoxy-Δ^5-pregnen-3β-ol-20-one	—	0.10	0.12	—	—
16α,17-Epoxy-3β-trichloroacetoxy-Δ^5-pregnen-20-one (I)	0.48	0.78	0.87	—	—
5α,6β,16β,21-Tetrabromide from I	—	0.11	0.42	—	—
16β-Bromo-21-iodo-3β-trichloroacetoxy-Δ^5-pregnen-17α-ol-20-one	—	0.21	0.49	—	—
16β-Chloro-Δ^5-pregnene-3β,17α-diol-20-one	—	0.04	—	—	—
16β-Chloro-3β-trichloroacetoxy-Δ^5-pregnen-17α-ol-20-one	—	0.23	—	—	—
21-Acetoxy-16α,17-epoxy-3β-trichloroacetoxy-Δ^5-pregnen-20-one	0.15	0.60	0.85	0.92	0.94
21-Acetoxy-16α,17-epoxy-Δ^5-pregnen-3β-ol-20-one	—	—	0.17	0.36	0.66

TABLE 12

R_M VALUES OF 6-OXYGENATED 4-EN-3-ONES FROM OXIDISED STEROIDS

(E. MENINI AND J. K. NORYMBERSKI, *Biochem. J.*, 84 (1962) 195)

Solvents: S_1 = Light petroleum–benzene–methanol–water (67:33:80:20, by vol.) (I. E. BUSH, *Biochem. J.*, 50 (1952) 370).
S_2 = 2,2,4-Trimethylpentane–2-methylpropan-2-ol–methanol–water (20:9:9:2, by vol.) (W. R. EBERLEIN AND A. M. BONGIOVANNI, *Arch. Biochem. Biophys.*, 59 (1955) 90).
Paper: Whatman No. 2 (descending) movement of solvent S_1 slowed by tapering end immersed in mobile phase.
Temperature of run: 22°.
Detection: D_1 = U.V. light (250 mμ).
D_2 = Alkali test: 2 *N* NaOH in 25% (v/v) ethanol followed by inspection in white light and U.V. light (360 mμ). U.V. inspection repeated after air drying at room temperature.
D_3 = Zimmermann test: 0.5% (w/v) ethanolic *m*-dinitrobenzene + aq. 5 *N* KOH (1:1) treatment, then heating to 60° (in oven); frequent inspection. Reagents were applied by a trough and a roller.

Compound	R_M*	
	S_1	S_2
Progesterone	−0.55	−0.12
6-Oxo-pregn-4-ene-3,6,20-trione	−0.10	0.58
6β-Hydroxypregn-4-ene-3,20-dione	0.41	0.55
6α-Hydroxypregn-4-ene-3,20-dione	0.66	0.79
Androst-4-ene-3,17-dione	−0.18	0.33
6-Oxo-androst-4-ene-3,6,17-trione	0.16	0.91
6β-Hydroxyandrost-4-ene-3,17-dione	0.72	0.87
6α-Hydroxyandrost-4-ene-3,17-dione	1.13	1.19

* $R_M = \log (1/R_F - 1)$.

TABLE 13

R_F VALUES OF RIBOFLAVIN METABOLITES

(E. C. OWEN, *Biochem. J.*, 84 (1962) 96P)

Solvents: S_1 = *n*-Butanol system (J. L. CRAMMER, *Nature*, 161 (1948) 349).
S_2 = Isoamyl alcohol, water saturated.
Paper: *cf.* E. C. OWEN, J. P. MONTGOMERY AND R. PROUDFOOT, *Biochem. J.*, 82 (1962) 8P (descending).
Detection: U.V. light.

Compound	R_F	
	S_1	S_2
9-(2′-Hydroxyethyl)-6,7-dimethylisoalloxazine (I)	0.59–0.63	0.42–0.43
Acetyl derivative of I	0.70–0.73	0.54–0.56
8-(2′-Hydroxyethyl)-6,7-dimethyllumazine	0.47	0.81
6,7-Dimethyl-8-D-ribityllumazine	0.30	0.81

TABLE 14

R_F VALUES OF SOME TULIP PETAL ANTHOCYANINS

(A. H. HALEVY, *Biochem. J.*, 83 (1962) 637)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase).
S_2 = Butan-1-ol–2 *N* HCl (1:1, v/v; upper phase).
S_3 = 1% (w/v) HCl.
S_4 = Water–acetic acid–12 *N* HCl (85:15:3, by vol.).
S_5 = *m*-Cresol–acetic acid–water (25:1:24, by vol.; upper phase).
S_6 = Phenol–water (73:27, w/v).
S_7 = Ethyl acetate–*tert.*-butanol (commercial)–acetic acid–water (5:4:1:3, by vol.).
S_8 = 15% (v/v) acetic acid.
S_9 = 5% (v/v) acetic acid.
S_{10} = Propan-2-ol–2 *N* HCl (1:1, v/v).
S_{11} = Water.
Paper: Whatman No. 3 MM (presumed) (descending).
Time of run: 8 h (S_3,S_8,S_9); 16 h (S_1,S_2,S_4,S_6,S_7,S_{10}); 24 h (S_5).
Detection: Visible light.

Compound	R_F											λ_{max} ($m\mu$)
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	
Delphinidin-3-glucoside	0.24	0.12	0.05	0.18	0.17	0.44	0.23	0.27	—	—	—	552
Delphinidin-3-rhamnoglucoside	0.28	0.14	0.12	0.36	0.36	0.44	0.23	0.43	—	—	—	552
Pelargonidin-3-glucoside	0.44	0.39	0.15	0.38	0.66	0.78	0.47	0.56	0.40	0.40	0.21	518
Pelargonidin-3-rhamnoglucoside	0.38	0.32	0.24	0.47	0.65	0.77	0.39	0.62	0.48	0.52	0.29	518
A pelargonidin-3-rhamnoglucoside isomer*	0.41	0.36	0.29	0.54	0.63	0.77	0.38	0.70	0.57	0.59	0.40	518
Unknown acylated pigment	0.68	0.64	—	—	0.66	0.97	0.66	0.78	—	—	—	519

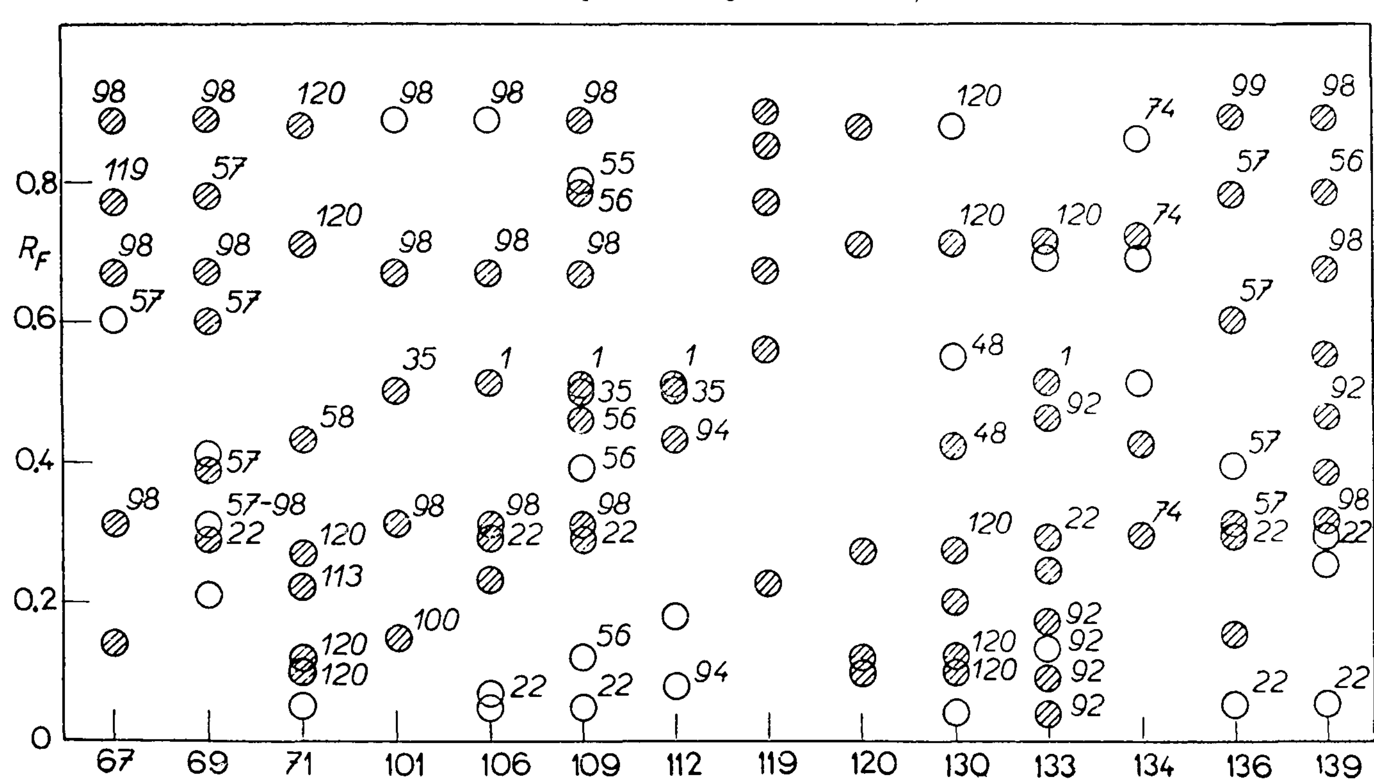
* Not more closely identified.

TABLE 15

R_F VALUES OF DYES PRESENTED IN MAP FORM

(J. GASPARIČ AND I. GEMZOVÁ-TABORSKÁ, *Collection Czech. Chem. Commun.*, 27 (1962) 2996)

Solvents: S_1. (See Tables 16–24 for details; the numbers correspond to those given in the tables.)



TABLES 16–24

R_F VALUES OF A SERIES OF COMMERCIAL DYESTUFFS

(J. GASPARIČ AND I. GEMZOVÁ-TÁBORSKÁ, *Collection Czech. Chem. Commun.*, 27 (1962) 2996)

Solvents: S_1 = pyridine–water (1:1); S_2 = pyridine–water (2:1); S_3 = 90% acetic acid; S_4 = ethanol–ammonia (1:1); S_5 = ethanol–1 *N* HCl (1:1); S_6 = hexane–benzene (2:1); S_7 = benzene; S_8 = benzene–chloroform.
(5–60 μg of substance applied where S_1 and S_2 used; for other solvents smaller amounts were taken.)

Paper: Whatman No. 3 (descending).

Impregnation: I_1 = 1-bromonaphthalene (10% in chloroform) (S_1–S_3); I_2 = lauryl alcohol (5% in ethanol) (S_4, S_5); I_3 = formamide (20% in ethanol) (S_6–S_8).
(Dip application; air-dried after application to remove solvent; where I_1 and I_2 used solvent saturated with impregnation agent to prevent its elution during run.)

Detection: Visible light.

Notes for Tables 16–24

[a] Dyes are numbered in the following order: yellow, orange, red, violet, blue, brown, green, black and gray. A small letter after the number indicates that it can only be differentiated from others of like number in the presence of another component.

[b] The commercial names have been left in the language of origin. + before the name of the dye implies that the structure of this product was hitherto unknown, although the chromatographic identity with other dyes of known structure had been established.

[c] Acna = Aziende Colori Nazionali Affini A. C. N. A., Milan; BASF = Badische Anilin und Soda Fabrik A. G., Ludwigshafen am Rhein; Bayer = Farbenfabriken Bayer A. G., Leverkusen; Delft = Nederlandsche Verf en Chemicalien Fabriek, N.V., Delft; Du Pont = E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.; Geigy = Geigy J. R., A. G., Basle; Hoechst = Farbwerke Hoechst A. G., Ffm. Hoechst; ICI = Imperial Chemical Industries Ltd., Manchester; Sandoz = Sandoz A. G., Basle; SSSR = Soviet dyestuffs; YDC = Yorkshire Dyeware & Chemical Co., Ltd., Leeds.

[d] The structures given are taken from the literature (*Colour Index*, 2nd Ed., Society of Dyers and Colourists, Bradford, 1956).

[e] Colours given are those obtained in the solvents given in the first column *i.e.*, S_1 or S_2. Bl = blue; Br = brown; Gr = green; Gy = gray; O = orange; Re = red; Ro = rose; V = violet; y = yellow.

[f] R_F values of the weaker spots are given in parenthesis. In systems S_3–S_8 these weak spots are not given in sequence. s = streaks.

[g] The identification of components in mixed dyestuffs are denoted by the number from the first column and this is based only on the R_F values in S_1 or S_2.

* Indicates that the structure was determined at the Research Institute for Organic Synthesis, Pardubice-Rybitví, Czechoslovakia.

TABLE 16

R_F VALUES OF YELLOW DYES

(For notes see p. D10)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f] S_2	S_3	S_4	S_5	S_6	S_7
1	Setacylgelb 2 GN	Geigy	11 855	Y	0.65	0.73	0.58	0.22	s	0.79
	+ Novalonechtgelb GR	Geigy								
	Serisol Fast Yellow GD	YDC								
	Cibacetgelb 2 GC	Ciba								
	+ Cibacetgelb GGR	Ciba								
	+ Delfacetechtgelb GSD	Delft								
	+ Delfacetechtgelb GD	Delft								
	+ Perlitongelb G	BASF								
	+ Palanilgelb G	BASF								
	Cellitonechtgelb G	BASF								
	+ Cellitonechtgelb GGR	BASF								
	Structure: $CH_3CONH-C_6H_4-N=N-C_6H_3(OH)(CH_3)$									
1a	+ Artisilgelb 2 GN	Sandoz	—	Y Y Y	0.65 (0.46) (0.52)	0.73 (0.62)	0.58 (0.41) (0.47)	0.22	s (0.47)	0.79
1b	+ Perlitongelb GR	BASF	—	Y Y O	0.65 (0.46) (0.49)	0.73 (0.66)	0.58	0.22	s (0.33)	0.79 (0.87)
1c	+ Cellitonechtgelb GR	BASF	—	Y O	0.65 (0.49)	0.73	0.58 (0.47)	0.22	s (0.33)	0.79
2	Artisildirectgelb GN Cibacetgelb GN	Sandoz Ciba	—	Y	0.47	0.56	0.79	0.44	0.06	—

(continued on p. D12)

TABLE 16 (*continued*)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f]					
					S_2	S_3	S_4	S_5	S_6	S_7
3	+ Resolingelb 5 R Cellitonechtgelb 5 R + Palanilgelb 5 R + Sholty protschny 4 K + Samarongelb 5 RL	Bayer BASF BASF SSSR Hoechst	26 090	Y	0.21	(0.03) 0.33	0.68	0.03	0.65	—
		Structure:			C_6H_5–N=N–C_6H_4–N=N–$C_6H_3(CH_3)$–OH					
3a	+ Setacylgelb 3 RN	Geigy	—	Y Y	0.21 (0.06)	(0.03) 0.33	0.68	0.03	0.65	—
4	Cellitonechtgelb 3 G	BASF	—	Y	0.22	0.18 (0.37)	0.46 0.89	0.13	0.90	—
5	Cellitonechtgelb 7 G	BASF	—	Y	0.19	s	s	s	0.90	—
6	Setacylgelb P-2 GL Setacylgelb P-GSL Novalongelb GSL Forongelb 2 GL	Geigy Geigy Geigy Sandoz	—	Y Y	0.36 (0.21)	0.44 0.54	— —	— —	0.00 0.60 (0.76)	0.91 —
7	Cibacetgelb GWL	Ciba	—	Y	0.37	0.52	—	—	0.36	0.88
8	Dispersol Fast Yellow A Cellitonechtgelb RR + Sholty protschny 2 K	ICI BASF SSSR	10 345	Y	0.29	0.70	0.78	0.30	0.01	—
			Structure:		O_2N–$C_6H_3(NO_2)$–NH–C_6H_4–OH					
9	Cibacetbrillantgelb 4 G	Ciba	—	Y	0.21	0.15 (0.20)	0.20	0.10	0.90	—
10	Serisol Fast Yellow GWD	YDC	—	Y	0.48	0.56	0.57	0.09	s	0.85

11	Cellitonechtgelb R	BASF	—	Y	0.48	0.65	0.41	0.61	0.89	0.95
12	Samarongelb RRL	Hoechst	—	Y	0.60	0.70	—	—	0.02	0.22
13	Cibacetgelb GR	Ciba	—	Y-O Y O Y Y	0.05 0.12 (0.29) 0.36 0.53	0.02 0.12 0.34 0.41 0.71	0.00 0.15 0.33 0.46 0.81	0.00 0.19 0.27 0.69	0.00 0.07 0.17 0.25 0.41 (0.56)	— — — — —
14	Resolingelb 4 G	Bayer	—	Y O-Re	0.26 0.65	0.27	0.88	s	0.89	—
15	+ Resolingelb 5 GS	Bayer	12 790	Y	0.45	0.37	0.75	0.24	s	—
	+ Samarongelb 5 G Cellitongelb 5 G + Palanilgelb 5 G + Giallo Neosetile 5 G	Hoechst BASF BASF Acna						0.30		
			Structure: O_2N–C_6H_4–N=N–(4-hydroxy-1-methyl-2-quinolone, 3-yl): OH, O, N–CH_3							
16	Resolingelb RL	Bayer	—	Y	0.21	0.20	0.73	0.07	0.90	—
			Structure*: CH_3COO–C_6H_4–N=N–C—C–CH_3 (HO–C, N, N–C_6H_5 pyrazole ring)							
17	Foronbrillantgelb 6 GFL	Sandoz	—	Y	0.32	(0.40) 0.50	—	—	s	0.91
18	Setacylgelb 3 G	Geigy	—	Y Y	0.39 0.30	(0.16) 0.43 (0.53)	— —	— —	0.00 0.77 (0.86)	0.95

(continued on p. D14)

TABLE 16 (*continued*)

No.[a]	*Dye*[b]	*Manufacturer*[c]	*C.I. No.*[d]	*Colour*[e]	R_F[f]					
					S_2	S_3	S_4	S_5	S_6	S_7
19	Setacylgelb P-5 G Setacylgelb 8 G	Geigy Geigy	—	Y	0.37	0.47	0.61 0.66	s	0.00 0.64	0.40
19a	Setacylgelb 5 G Artisilgelb 5 GP	Geigy Sandoz	—	Y Y	0.37 (0.28)	0.47 (0.17) 0.38	0.61 0.66	s	0.00 0.64 (0.75)	0.40
20	Duranol Brilliant Yellow 6 G + Palanilbrillantgelb 8 G	ICI BASF	58 900	Y	0.39	0.24	—	—	0.84	0.94
	Structure: (anthrone-type ring structure with OCH_3 and O)									
21	Cibacetgelb 5 GN Artisilgelb 5 GN	Ciba Sandoz	—	Y	0.36	0.26	0.72	0.54	0.75	0.95

TABLE 17

R_F VALUES OF ORANGE DYES

(For notes see p. D10)

No.[a]	*Dye*[b]	*Manufacturer*[c]	*C.I. No.*[d]	*Colour*[e]	R_F[f]					
					S_2	S_3	S_4	S_5	S_6	S_7
22	Artisilorange 2 R Serisol Fast Orange GD Cibacetorange 2 R Cellitonechtorange GR + Palanilorange GR Neosetile Orange GR + Oranshevyi Sh	Sandoz YDC Ciba BASF BASF Acna SSSR	11 005	O O	0.49 (0.11)	(0.71)	(0.10) 0.46	0.64	s	0.87

	Structure: O_2N–⌬–N=N–⌬–NH_2									
23	Dispersol Fast Orange B + Samaronorange B + Palanilorange G	ICI Hoechst BASF	26 080	O	0.19	(0.04) 0.30	0.56	0.02 (0.50)	0.60	0.90
	Structure: ⌬–N=N–(naphthalene)–N=N–⌬–OH									
24	Duranol Orange G Artisilorange 3 RP	ICI Sandoz	60 700	O O Re Y	(0.29) 0.47 (0.60) (0.65)	0.58	—	—	0.75	0.93
	Structure: (anthraquinone: O, O, NH_2, CH_3)									
25	Resolinorange GL	Bayer	—	O	0.24	0.24	—	—	—	0.95
	Structure*: (anthraquinone: O, O, OH, OH)									
26	Setacylorange G Novalonorange G	Geigy Geigy	—	O	0.32	0.77	0.31 0.37	s	0.80	0.94
27	+ Resolinorange 5 R	Bayer	11 080	O	0.13	0.19	0.16	0.09	0.81	0.94
27a	+ Palanilorange 5R	BASF		O V V	0.13 (0.08) (0.25)	0.19	0.16	0.09	0.81	0.94

(continued on p. D16)

TABLE 17 (*continued*)

No.[a]	*Dye*[b]	*Manufacturer*[c]	*C.I. No.*[d]	*Colour*[e]	R_F[f] S_2	S_3	S_4	S_5	S_6	S_7
27b	Cellitonechtorange 5 R	BASF		O V V	0.13 (0.08) (0.58)	0.19	0.16	0.09	0.81	0.94
	Structure: $O_2N-C_6H_4-N=N-C_6H_4-NH-C_6H_5$									
28	Setacylorange 2 R Artisilorange 4 RP	Geigy Sandoz	—	Y Re-O	0.24 0.38	0.71 0.76	(0.06) 0.30 0.37	s (0.77)	0.61	0.93
29	Foronbrillantorange GL	Sandoz	—	O	0.32	0.18	s	s	0.91	—
30	Oranschevy 2 K	SSSR	—	Re-O	0.56	0.82	0.51	0.73	—	—
	Structure: $Cl-C_6H_3(NO_2)-N=N-C_6H_3(CH_3)-N(CH_2CH_2OH)_2$									
31	Cellitonechtorange GF 2 R	BASF	—	Re-O Re-O	0.55 0.39	0.50 0.76	0.50 0.57	0.48 0.63	0.03 0.42	—
32	Foronorange GFL Artisilorange GFL	Sandoz Sandoz	—	O Y	0.25 (0.36)	0.48	0.68	0.08	s	—
	Structure*: $C_6H_5-N=N-C_6H_2(CH_3)(OCH_3)-N=N-C_6H_4-OH$									
33	Foronorange RFL	Sandoz	—	Re Re-O Y	(0.06) 0.26 (0.36)	0.58	0.73 (0.83)	0.16 (0.45)	s	—

TABLE 18

R_F VALUES OF RED DYES

(For notes see p. D10)

No.[a]	*Dye*[b]	*Manufacturer*[c]	*C.I. No.*[d]	*Colour*[e]	R_F[f]						
					S_2	S_3	S_4	S_5	S_6	S_7	S_8
34	Samaronrosa RFL Perlitonbrillantrosa R Palanilrosa RF	Hoechst BASF BASF	—	Ro	0.48	0.57 (0.65)	0.01	0.27	0.71	0.93	0.90
35	Serisol Fast Red 2 RDX Cibacet Rot 2 G + Perlitonrot 3 B + Palanilrot GG Cellitonechtrot GG	YDC Ciba BASF BASF BASF	11 210	Re	0.64	0.91	0.52	0.77	0.00	0.41	0.57
	Structure: $O_2N-C_6H_4-N{=}N-C_6H_3(CH_3)-N(CH_2CH_2OH)_2$										
36a	Cibacetbordeaux NH	Ciba	—	Re Re Re Ro	0.32 0.64 (0.11) (0.42)	0.91 0.65 (0.82)	—	0.44 0.77	0.00 0.44 (0.31)	0.41 0.91	0.57 0.92
36b	Cellitonechtrot BB	BASF	—	Re	0.32	0.91	—	0.44	0.00	0.41	0.57
				Re	0.64	0.65		0.77	0.44	0.91	0.92
				Y	(0.11)		0.52	(0.64)			
				Re	(0.50)		0.29	(0.85)			
				Ro	(0.72)		(0.41)				
	Structure*: mixture of 35 and 37										
37	Cibacetrubin R + Artisilrubin GP Cellitonechtrubin B + Bordo S	Ciba Sandoz BASF SSSR	11 115	Re	0.32	0.65	—	0.44	0.44	0.91	0.92

(continued on p. D18)

TABLE 18 (*continued*)

No.[a]	*Dye*[b]	*Manufacturer*[c]	*C.I. No.*[d]	*Colour*[e]	R_F[f]						
					S_2	S_3	S_4	S_5	S_6	S_7	S_8
37a	+ Delfacetechtrot AD	Delft	—	Re Re Ro Re	0.32 (0.11) (0.42) 0.74	0.65 (0.22) (0.82) 0.87	— (0.16) 0.29 (0.40) 0.58	0.44 (0.57) 0.76 —	0.44 0.00 (0.31) —	0.91 0.30 — —	0.92 0.48 — —
	Structure: $O_2N-C_6H_3(Cl)-N{=}N-C_6H_4-N(C_2H_5)CH_2CH_2OH$										
38	Setacylrosa BG	Geigy	—	Re Re	s 0.76	0.94	—	(0.49) 0.74	0.00 (0.06)	s s	0.28 (0.43) (0.50)
39	Setacylbrillantrot P-BL Samaronrosa BRL Palanilrosa BF Novalonbrillantrot BL	Geigy Hoechst BASF Geigy	—	Ro	0.73	0.82	—	0.38 (0.09)	0.00 0.02 (0.85)	s	0.00 0.47
39a	Cibacetbrillantrosa FG	Ciba	—	Ro Ro Ro	0.73 (0.26) (0.96)	0.82	—	0.38 (0.09)	0.00 0.02 (0.85)	s	0.00 0.47
	Structure*: anthraquinone, 1-NH_2, 2-OCH_2CH_2OH, 4-OH										
40	Resolinrot FB	Bayer	—	Ro	0.22	0.42	s	s	0.93	0.95	0.95
	Structure*: anthraquinone, 1-NH_2, 2-O-C_6H_5, 4-OH										

41	Cibacetscharlach B$\overline{R}$	Ciba	—	Re Ro	0.21 (0.45)	0.60 (0.98)	0.27	0.67 0.71	0.93	0.95	0.92
42	Serisol Brilliant Red X 3 B Duranol Red X 3 B Cellitonechtrosa FF 3 B	YDC ICI BASF	62 015	Ro	0.75	0.81	—	0.28 0.45	0.09	0.64	0.74
42a	Cibacetbrillantrosa 4 BN Artisilbrillantrosa 5 BP	Ciba Sandoz		Ro Bl	0.75 (0.43)	0.81 (0.60)		0.28 0.45	0.09	0.64	0.74
	Structure: 1,4-diamino-2-methoxyanthraquinone (O, NH_2, OCH_3, O, NH_2)										
43	+ Setacylscharlach GRN Cibacetscharlach 2 B + Delfacetechtscharlach BD + Artisilscharlach GFP + Aly Sh Cellitonscharlach B	Geigy Ciba Delft Sandoz SSSR BASF	11 110	Re	0.49	0.85	0.40	0.73	0.32	0.88	0.89
	Structure: $O_2N-C_6H_4-N{=}N-C_6H_4-N(CH_2CH_3)(CH_2CH_2OH)$										
44	Setacylrubine B + Samaronbordo 3 B + Palanilrubin 3 BD Cellitonechtrubin 3 B	Geigy Hoechst BASF BASF	11 215	O Re Re	(0.09) (0.41) 0.53	0.80	—	0.61	0.03	0.55	0.67
	Structure: $O_2N-C_6H_3(Cl)-N{=}N-C_6H_3(CH_3)-N(CH_2CH_2OH)_2$										
45	Setacylrosa 3 BN Serisol Fast Red 2 B Duranol Red 2 B Artisilrot 3 BP + Krasny 2 S	Geigy YDC ICI Sandoz SSSR	60 710	Re	0.54	0.72	0.55	0.32	0.61	0.87	0.87

(continued on p. D20)

TABLE 18 (*continued*)

No.[a]	*Dye*[b]	*Manufacturer*[c]	*C.I. No.*[d]	*Colour*[e]	R_F[f] S_2	S_3	S_4	S_5	S_6	S_7	S_8
45a	Cellitonechtrosa BN	BASF	—	Re Re	(0.48) 0.54	—	—	—	—	—	—
	Structure: 1-amino-4-hydroxyanthraquinone (O, NH_2, O, OH)										
46a	Setacylrot GBN	Geigy	—	Re	0.49	0.85	0.49	0.79	0.17	0.86	0.88
46b	Palanilrot 4 G Cellitonechtrot 4 G	BASF BASF	—	Re Re	(0.49) 0.28	(0.85) 0.60		(0.79) 0.75	(0.17) 0.84	(0.86) 0.94	(0.88) 0.94
47a	+ Palanilscharlach R	BASF	11 150	Y O Re Ro	(0.32) (0.50) 0.62 (0.42)	0.86	0.49 (0.40)	0.58	0.02	0.41	0.54
47b	Cellitonechtscharlach R	BASF	—	Y O Re Re	(0.32) (0.50) 0.62 (0.69)	0.86	0.49	0.58	0.02	0.41	0.54
	Structure: $O_2N-C_6H_4-N{=}N-C_6H_3(Cl)-N(CH_2CH_2OH)_2$										
48	Setacylscharlach 2 GN Setacylscharlach P-2 G Novalonscharlach G Novalonscharlach 2 GN	Geigy Geigy Geigy Geigy	—	Re Y	0.59 0.70	0.88 (0.90)	0.45 (0.75)	s	0.00 0.07	0.13 0.27 0.69	0.25 0.37 0.76
49	Resolinrot 3 BL	Bayer	—	Re	0.21	0.36	—	s	0.88	0.95	0.95

Structure*: anthraquinone with O, NH_2, Br, O, OH

50	Setacylrot P-2 GL Artisilscharlach GFL Samaronscharlach 3 R	Geigy Sandoz Hoechst	—	O Y O-Re Re Re Re	(0.09) (0.21) 0.32 0.45 (0.58) (0.69)	0.82 (0.68) (0.60) (0.09)	(0.10) (0.34) 0.45	0.28	s 0.36	0.77 0.93	0.93 s

Structure*:

$O_2N-C_6H_3(Br)-N=N-C_6H_4-N(CH_2CH_2OCOCH_3)(CH_2CH_2CN)$

$O_2N-C_6H_3(Br)-N=N-C_6H_4-N(CH_2CH_2OH)(CH_2CH_2CN)$

51a	Foronscharlach 3 GFL	Sandoz	—	O-Re	0.32	0.65	—	0.28	0.61	0.94	0.93
51b	Cibacetscharlach B	Ciba	—	O-Re Re	(0.32) 0.44	(0.65) 0.82	— (0.34) 0.45	0.28	(0.61) s	(0.94) 0.77	(0.93) 0.83
51c	Palanilscharlach RR	BASF	—	O-Re Re	0.32 0.44	0.65 0.82	0.45 0.34	0.28	0.61 s	0.94 0.77	0.93 0.83

Structure*:

$O_2N-C_6H_3(Cl)-N=N-C_6H_4-N(CH_2CH_2OCOCH_3)(CH_2CH_2CN)$

$O_2N-C_6H_3(Cl)-N=N-C_6H_4-N(CH_2CH_2OH)(CH_2CH_2CN)$

(continued on p. D22)

TABLE 18 (*continued*)

No.[a]	*Dye*[b]	*Manufacturer*[c]	*C.I. No.*[d]	*Colour*[e]	R_F[f]						
					S_2	S_3	S_4	S_5	S_6	S_7	S_8
52	Palanilrot 5 G	BASF	—	O	0.09	0.28	0.17	0.00	0.02	0.81	0.91
				Ro	0.13	0.33	0.23	0.02	0.05	0.94	0.94
				Re	0.19	0.37	0.31	0.06	0.11		
				Re	0.32	0.50	0.36	0.16	0.42		
				Re	0.42	0.71	0.45	0.29	0.55		
				Re	0.56	0.76		(0.34)	0.75		
				Y	0.74	0.89		(0.46)	0.91		
				Y	0.84			(0.90)			
				Y	0.92						
53	Serinyl Brilliant Scarlet RD	YDC	—	O	0.83	0.94	—	0.62	0.00	0.01	0.01
				Re	0.87			(0.78)		0.05	(0.05)
				Y	0.92						
54	Palanilrosa BBD	BASF	—	V-Re	0.62	—	—	(0.50)	—	0.76	—
	Rubinovy S	SSSR						0.63		(0.89)	
55	Cibacetbrillantscharlach RG	Ciba	—	Re	0.82	0.94	0.80	0.90	0.00	s	0.49

Structure*: CH_3SO_2–C_6H_3(Cl)–N=N–C_6H_3(CH_3)–$N(CH_2CH_2OH)_2$

TABLE 19

R_F VALUES OF VIOLET DYES

(For notes see p. D10)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f]					
					S_1	S_3	S_4	S_5	S_6	S_7
56	Setacylviolett R Violetto Neosetile 3 R Cibacetviolett 2 R Serisol Brilliant Violet 2 R Duranol Violet 2 R Artisilviolett 2 RP + Perlitonviolett 3 R	Geigy Acna Ciba YDC ICI Sandoz BASF	61 100	V Ro	0.78 (0.39)	(0.61) 0.73	0.47 0.56	0.60	0.06 (0.55)	0.45 (0.85)
56a	+ Setacylviolett P-R	Geigy	—	V Ro Y	0.78 (0.39) (0.87)	—	—	—	—	0.45 (0.85)
56b	Cellitonechtrotviolett RN	BASF	—	V Ro Ro Bl	0.78 (0.39) 0.10 0.14	—	0.47 0.57 0.27 0.37	0.60 0.12 0.14	—	0.45 (0.85) 0.93
56c	+ Cibacetviolett RB	Ciba	—	V Ro Bl Bl	0.78 (0.39) 0.12 0.46	—	—	—	0.06 (0.55) 0.93 0.74	0.45 (0.85) 0.90

Structure: (1,4-diaminoanthraquinone: O, NH2, O, NH2)

(continued on p. D24)

TABLE 19 (*continued*)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f] S_1	S_3	S_4	S_5	S_6	S_7
57	+ Setacylviolett B Novalonviolett B Cibacetviolett B Serisol Fast Violet 6 B Duranol Brilliant Violet B Artisilviolett BP	Geigy Geigy Ciba YDC ICI Sandoz	61 105	Bl V	0.60 (0.78)	0.69 0.73	—	0.61	0.06 0.43	0.82
57a	Cellitonechtviolett 6 B	BASF	—	Bl V V Ro	0.60 0.78 0.31 0.39	0.69 0.73 0.48 0.61	 0.47 0.57 0.65	0.61	0.06 0.43 (0.55) (0.80)	0.82 0.45 (0.92)

Structure: O NH_2 O $NHCH_3$

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	S_1	S_3	S_4	S_5	S_6	S_7
58	Setacylviolett BR	Geigy	—	Bl V	(0.29) 0.43	0.80	—	s	0.00	s
59	Duranol Brilliant Violet BR + Cibacet Brilliant Violet 3 B	ICI Ciba	62 030	Bl-V	0.72	0.71	—	s	0.00	0.23
59a	+ Samaronbrillantviolett B + Palanilviolett 3 B Cellitonechtviolett B	Hoechst BASF BASF	—	Bl-V V	0.72 (0.78)	0.71	 0.61	s	0.00	0.23

Structure: O NH_2 O_2N O NH_3

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	S_1	S_3	S_4	S_5	S_6	S_7
60	Duranol Violet RN	ICI	—	Ro Bl-V Ro	(0.62) 0.82 (0.86)	—	—	0.45	0.00	0.03 0.23

61	+ Setacylviolett P-4 RT Setacylviolett 4 RT + Fioletovy 4 K	Geigy Geigy SSSR	11 120	Ro Ro Ro V	(0.08) (0.14) (0.25) 0.38	0.81 0.85	—	(0.38) 0.74 (0.81)	0.00 (0.03)	s

Structure: $O_2N-C_6H_3(NO_2)-N{=}N-C_6H_4-N(CH_2CH_2CH_2CH_3)(CH_2CH_2OH)$

62	Setacylviolett 2 R	Geigy	—	V V	(0.38) 0.56	0.81 (0.85)	—	(0.24) 0.66	0.00	s (0.12)
63	Cibacetviolett 5 R Palanilviolett 5 R	Ciba BASF	—	O-Re V V	(0.08) 0.16 (0.46)	(0.39) 0.48 (0.76)	—	s	0.58	s
64	Celliton Violet R	BASF	11 195	V Y O-Re	0.09 0.19 0.24	0.68	—	(0.04) (0.40) 0.64	s	s

Structure: $O_2N-C_6H_3(NO_2)-N{=}N-C_6H_3(CH_3)-N(CH_2CH_2CH_2CH_3)(CH_2CH_2OH)$

65	Resolinviolett RL Palanilviolett R	Bayer BASF	—	V	0.13 s	(0.24) 0.33	s	0.00 (0.10) (0.13)	0.01 0.78 (0.89)	s

Structure*: 1,4-diamino-2-bromoanthraquinone (O, NH_2, Br, O, NH_2)

66	Foronbrillantviolett BL	Sandoz	—	Bl V	0.06 0.38	0.20	s	0.00 0.04	0.93	0.94

TABLE 20

R_F VALUES OF BLUE DYES

(For notes see p. D 10)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f]						Components[g]
					S_1	S_3	S_4	S_5	S_6	S_7	
67	Cellitonechtmarineblau BF	BASF	—	Re	0.14	0.48	—	0.40	0.00	0.02	
	Cellitonechtmarineblau BN	BASF		Bl	0.31	0.61		0.45	0.09	(0.09)	57; 98
				Bl	(0.60)	0.71		0.61	0.61	(0.30)	57
				Bl	0.67	0.79		0.75	0.88	0.51	98
				Gr-Bl	0.77			(0.83)		(0.87)	119
				Bl	0.89					0.94	98
68	Perlitonmarineblau BR	BASF	—	Br	0.14	0.48	—	0.00	—	0.33	
	Cellitonechtmarineblau BR	BASF		Y	0.25	0.51		0.30		0.46	
				V	(0.31)	0.60		0.40		(0.82)	57
				Ro	0.39	(0.65)		0.45		0.87	57
				Bl	0.60	0.72		0.61		(0.94)	57
				V	0.78	0.73					57
				Bl	0.87						75
68a	Cellitonechtmarineblau BGN	BASF	—	Y	0.14	—	—	—	—	—	
				V	(0.31)	0.48		0.00			57
				Ro	0.39	0.65		0.34			57
				Bl	0.60	0.72		0.40			57
				V	0.78	0.73		0.61			57
				Bl	0.87						75
69	Cibacetmarineblau RNJ	Ciba	—	Bl	(0.21)	(0.48)	—	0.34	—	0.02	
				O	0.29	0.60		0.58		0.46	22
				V	(0.31)	0.72		0.70		0.50	57; 98
				Ro	0.39	0.82				0.66	57
				Bl	(0.41)					(0.82)	
				Bl	0.60					(0.88)	57
				Bl	0.67					(0.94)	98
				V	0.78						57
				Bl	0.89						98
70	Cellitonechtmarineblau GTN	BASF	—	Bl	0.09	(0.48)	—	0.30	—	0.02	
				Br-O	0.18	0.61		(0.45)		0.09	
				Bl	0.21	0.65		0.60		0.46	
				V	0.31	0.69		(0.70)		0.82	57
				Ro	(0.39)	0.78				0.88	57

				Bl	0.60					0.94	57
				Bl	(0.62)						
				Gr-Bl	0.74						
				V	0.78						57
				Bl	0.87						75
71	Setacylmarineblau 2 B	Geigy	—	Gr	0.10	(0.16)	—	0.09	—	0.00	120
				Bl	0.12	0.34		(0.13)		0.13	120
				O	0.22	(0.40)		0.28		0.19	113
				Bl	0.27	0.46		0.34		0.75	120
				V	0.43	0.60		0.48		(0.83)	58
				Gr	0.71	(0.66)		0.65		0.89	120
				Bl	0.88	0.83				(0.93)	120
71a	Setacylmarineblau BTA	Geigy	—	Gr	0.05	—	—	—	—	—	
	Setacylmarineblau RA	Geigy		Gr	0.10	(0.16)		0.09		0.00	120
				Bl	0.12	0.34		(0.13)		0.13	120
				O	0.22	(0.40)		0.28		0.19	113
				Bl	0.27	0.46		0.34		0.75	120
				V	0.43	0.60		0.48		(0.83)	58
				Gr	0.71	(0.66)		0.65		0.89	120
				Bl	0.88	0.73				(0.93)	120
72	Setacylmarineblau TS	Geigy	—	Gr	(0.10)	(0.34)	—	0.00	0.00	0.00	120
				Bl	0.12	0.77		0.30	(0.21)	0.05	120
				V	(0.38)	0.81		0.56		0.08	62
				V	0.56			0.62		(0.23)	62
				Gr	0.71			0.71		(0.75)	120
				V	0.78						56
				Bl	0.87						75
				Bl	0.88						120
73	Cellitonechtblau FW	BASF	61 510	V	(0.30)	0.74	—	0.76	—	0.00	
				Bl	0.71	0.85		0.87	—	(0.12)	
				Bl	0.97						

Structure: O NHCH$_3$ / O NHCH$_2$CH$_2$OCH$_2$CH$_2$OH

(continued on p. D28)

TABLE 20 (*continued*)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f] S_1	S_3	S_4	S_5	S_6	S_7	Components[g]
74	Cellitonechtmarineblau B	BASF	—	Re	0.19	0.70	—	0.00	—	0.00	
				O	(0.29)	(0.78)		0.29		0.09	22
				V	0.72			0.61		0.25	
				Bl	0.87			0.80			75
75	Cibacetsaphirblau G	Ciba	64 500	Bl	0.87	0.77	0.61	s	—	0.00	
	Artisilblau SAP	Sandoz				(0.85)	(0.54)			0.09	
	+ Cellitonblau G	BASF								0.23	
	Duranol Brilliant Blue CB	ICI								(0.44)	
	Setacylblau 2 GS	Geigy									
			Structure:	H_2N O NH_2 / H_2N O NH_2							
76	Cibacetblau 5 G blauer	Ciba	—	Bl	(0.05)	0.79	—	—	0.00	0.00	
				Re	(0.11)	0.86			0.02	(0.02)	
				Bl	(0.18)					0.30	
				Gr	0.31					0.91	
				Gr	0.68						
				Bl	(0.87)						
				Bl	0.89						99
77	Samaronblau TGL	Hoechst	—	Bl	0.10	0.47	—	s	—	0.83	
				Bl	s	0.67					
				V	0.31						
78	Foronblau 3 RFL	Sandoz	—	V	0.09	(0.08)	—	0.01	s	0.72	
				O	0.12	0.16		0.04	0.95	0.94	
				V	0.29	(0.34)		0.17			
				Gr	0.65	0.71					
79	Resolinblau RRL	Bayer	—	V	0.09	0.01	—	0.04	0.00	0.00	
				O	(0.12)	0.14			0.95	0.95	
				Bl	(0.15)	0.34					
				V	0.96	0.81					
80	Cibacetblau F 3 GN	Ciba	—	Bl	0.10	0.25	—	0.08	0.44	0.82	
				Bl	0.24	0.65		0.18	0.95	0.94	

81	Cibacetblau RF	Ciba	—	Ro Gr Bl Bl Bl	(0.14) (0.24) 0.31 0.39 0.55	0.48 0.70 0.80	s 0.54	s 0.57	—	(0.06) 0.69 0.92	97
82	+ Palanilblau GR Cellitonechtblau FR	BASF BASF	61 115	Bl Bl Bl V Gr-Bl Bl	0.20 (0.52) (0.62) (0.72) 0.74 0.97	0.48 (0.05) (0.70) 0.78	0.04 0.49 0.59 0.69	0.27 (0.62)	—	0.02 0.09 0.24 0.93	
	Structure: O, NH_2, O, NH–, OCH_3										
83a	Cibacettürkisblau G	Ciba	—	Gr V Bl Bl Bl Gr-Bl	(0.87) (0.19) (0.22) (0.52) 0.62 0.74	0.78	0.59 0.69	0.65	—	0.02 0.10	
83b	Palanilblau 7 G Sine-seleny	BASF SSSR	—	Gr V Bl Bl Bl Gr-Bl	(0.97) (0.19) (0.22) (0.52) 0.62 0.74	0.78 (0.65) 0.69	—	—	—	—	
84	Setacylätzblau GNN Artisilätzblau GNN	Geigy Sandoz	—	Gr-Bl Bl Bl Bl Bl Bl Bl	0.06 0.11 0.26 0.39 0.55 0.74 0.86	0.37 0.51 (0.56) (0.64) 0.78 (0.85) 0.90	—	0.78 0.83	0.00 0.06 0.68 0.93	s 0.82 0.94	

(continued on p. D30)

TABLE 20 (*continued*)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f]						Components[g]
					S_1	S_3	S_4	S_5	S_6	S_7	
85	Cellitonätzblau 5 G	BASF	11 435	Bl Gr-Bl	0.07 0.70	(0.35) (0.40) 0.84	s	0.23	0.01	0.00 0.03	
	Structure:										
86	Cellitonätzblau GFR	BASF	—	Bl Gr V	(0.13) (0.57) 0.67	—	—	0.08 0.18 0.30 0.50	—	0.06 (0.08)	
87	Cellitonätzblau 3 G	BASF	11 430	V Gr Gr	0.07 0.22 (0.34)	(0.72) 0.88	—	0.45	0.00 0.04	0.35	
	Structure:										
88	Setacylblau P-RS	Geigy	—	Bl	0.22	0.48	—	0.20	—	(0.88) 0.93	
89	Setacylblau P-GFL Samaronblau 3 GL Artisilblau GFL Foronblau GFL Palanilblau 5 G	Geigy Hoechst Sandoz Sandoz BASF	—	Gr Bl Bl Bl V Bl O-Br	(0.19) (0.43) 0.52 0.72 0.78 (0.86) (0.88)	(0.61) 0.72	0.00 0.37 0.52 0.57	0.30 s	—	0.00 0.04 (0.20) 0.26	

90	Cellitonechtblau FFB	BASF	62 035	Gr-Bl Bl Bl Bl	(0.15) (0.31) (0.60) 0.78	(0.69) 0.76	0.57 0.65 0.72	0.52	—	0.13

Structure: O NH₂ CONH₂ O NHCH₃

91	Setacylblau P-4 GS Cibacetsaphirblau 4 G Artisilblau 4 GP	Geigy Ciba Sandoz	—	Bl-V Bl Bl Bl Gr-Bl	(0.21) 0.26 0.46 0.55 (0.74)	0.68 0.78	s	0.42	—	(0.06) 0.69 (0.90)
92	Cibacetmarineblau NH Cibacetmarineblau RL Cibacetdunkelblau RB	Ciba Ciba Ciba	—	Bl V Bl	0.09 0.17 0.46	0.30 0.65	—	0.05 0.12 0.35 0.40	—	0.66 (0.93)
93	Setacylätzblau 2 R Setacylblau 4 GS Artisilätzblau 2 R	Geigy Geigy Sandoz	—	Bl-V Bl Bl	0.26 (0.37) 0.49	0.82 0.91	—	s	—	s (0.36) (0.56)
94	+ Samaronblau FGD + Perlitonbrillantblau B + Palanilblau G Cellitonechtblau FFG	Hoechst BASF BASF BASF	62 050	Bl Bl	0.08 0.43	(0.29) 0.70	(0.10) 0.14 0.34 (0.50) (0.59)	0.10 0.40	—	0.57 (0.94)

Structure: O NH₂ CONH₂ O NHCH(CH₂–CH₂)₂CH₂ [cyclohexylamino: NHCH, CH₂–CH₂, CH₂, CH₂–CH₂]

95a	Duranolblau G	ICI	63 305	Bl Bl Bl	(0.10) 0.23 0.46	0.26 0.51 0.68	—	0.01 0.09 0.45	—	0.61 0.93

(continued on p. D32)

TABLE 20 (*continued*)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f] S_1	S_3	S_4	S_5	S_6	S_7	Components[g]
95b	+ Duranolblau GN	ICI	—	Bl Bl Bl Bl	0.31 (0.10) 0.23 0.46	—	—	—	—	—	
			Structure:	OH O NHCH$_3$ CH$_3$HN O OH							
96	Samaronblau FBL Resolinblau FBL Foronblau BL Palanilblau R	Hoechst Bayer Sandoz BASF	—	Bl Bl Bl Bl	0.09 0.15 (0.23) 0.46	0.10 0.17 0.27 0.46 0.57 0.68	—	0.02 0.11	—	0.59 0.85	
97	+ Setacylblau BS Duranol Brilliant Blue G Cellitonechtblau B	Geigy ICI BASF	61 500	Bl	0.31	(0.38) 0.48	—	(0.25) 0.60	—	0.93	
97a	Cibacetblau BR	Ciba	—	Bl V-Bl	0.31 0.17	—	—	—	—	—	
97b	Artisilblau BRP + Artisilblau ERP	Sandoz Sandoz	—	Bl Bl Bl	0.31 (0.03) 0.17	—	—	—	—	—	
			Structure:	O NHCH$_3$ O NHCH$_3$							
98	Cibacetblau F 3 R Cellitonechtblau FFR	Ciba BASF	61 505	Bl Bl	(0.05) (0.17)	0.48 0.71	0.47 0.65	(0.00) (0.25)	—	0.02 0.51	

	+ Siny K + Celanthrene Brilliant Blue	SSSR		Bl	0.31	0.81	0.72	0.60		0.93
	FFSK	Du Pont		Bl	0.67			0.75		
				Bl	0.89			(0.82)		

Structure: O, $NHCH_3$, O, $NHCH_2CH_2OH$

99	Cellitonechtblau BF	BASF	61 545	Bl	0.89	—	—	—	—	—

Structure: O, $NHCH_2CH_2OH$, O, $NHCH_2CH_2OH$

TABLE 21

R_F VALUES OF BROWN DYES

(For notes see p. D10)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f] S_1	S_3	S_4	S_5	S_6	S_7	Components[g]
100	Cibacetbraun 4 RN	Ciba	11 100	Br	0.15	0.60	0.30	0.28	0.68	0.89	
	Artisilbraun H	Sandoz					(0.20)	(0.40)			
	+ Palanilbraun 3 R	BASF									
	Cellitonechtbraun 3 R	BASF									
101	Cellitonechtbraun BT	BASF	—	Br	0.15	0.45	(0.20)	0.28	0.00	(0.02)	100
				Bl	0.31	0.60	0.30	(0.40)	0.04	0.38	97; 98
				Re	0.50	0.75	0.46	(0.61)	0.13	(0.58)	35
				Bl	0.67	0.79	0.63	0.70	0.68	0.89	98
				Bl	(0.89)	0.85	0.72	0.75	0.89		98
102	Cibacetbraun R	Ciba	—	Br	(0.05)	0.45		0.28	(0.32)	(0.75)	
				Br	0.15	0.60		0.40	0.68	0.89	100
				Bl	0.31			0.54	0.86	0.91	97
				Bl	(0.43)						
				Bl	(0.52)						
103	Cibacetbraun RBN	Ciba	—	Br	(0.05)	0.60	(0.20)	0.30	0.00	0.00	
				Bl	(0.08)	0.64	0.30	0.51	0.62	0.02	94
				Br	0.18	0.75	0.35	0.60	(0.75)	(0.73)	
				Re	0.23	0.80	0.46	0.75	(0.87)	0.85	
				Bl	0.43		(0.58)			0.89	94
				Bl	(0.74)		0.63				
							(0.80)				
104	Duranol Dark Brown B	ICI	—	Bl	(0.16)	(0.34)	0.35	0.28	0.00	0.85	
				Re	0.23	0.45	0.42	0.54	(0.15)	0.92	
				O	0.29	0.70	0.46	0.61	0.62		22
				Bl	(0.31)	0.85	(0.58)	(0.70)	0.86		98
				Bl	0.67		0.63	0.75			98
				Bl	(0.89)		(0.72)				98

Structure (No. 100): $O_2N-C_6H_2Cl_2-N=N-C_6H_4-N(CH_3)CH_2CH_2OH$ (2,6-dichloro-4-nitrophenyl azo)

105	Delfacetechtbraun BVC	Delft	—	Ro	(0.07)	(0.34)	(0.20)	0.30	(0.16)	0.78	
				Bl	(0.16)	0.45	0.35	0.54	0.46	0.85	
				Re	0.23	0.75	0.51	(0.70)	0.57	0.92	
				Bl	(0.31)	0.79	0.63	0.75	0.88		98
				Y	0.51				(0.93)		1
				Bl	0.67						98
				Bl	(0.76)						
				Bl	0.89						98
106	Cibacetbraun BNH	Ciba	—	O	(0.05)	(0.45)	(0.20)	0.30	(0.10)	0.02	22
				Ro	(0.07)	0.70	0.35	0.54	0.43	(0.56)	
				Re	0.23	0.75	0.42	0.61	0.52	0.78	
				O	0.29	0.79	(0.46)	(0.70)	0.58	0.85	22
				Bl	0.31		0.51	0.75	(0.86)	(0.92)	98
				Y	0.51		0.63				1
				Bl	0.67		(0.72)				98
				Bl	(0.89)						98
107	Serinyl Hosiery Brown RN	YDC	—	Y	0.50	0.79	0.51	0.30	s	0.02	
				Re	0.59	0.80	0.56	0.61	0.50	0.30	
				Bl	0.89		0.72	0.75		0.78	99
	Serisol Hosiery Brown GN	YDC	—	Bl	(0.09)	0.75	0.35				
				Re	0.23	0.79	0.51	0.30			
				Y	0.50	0.80	0.56	0.61			
				Re	0.59		0.72	0.75			
				Bl	0.89						99
108	Perlitonbraun GR	BASF	—	O	0.29	0.70	(0.18)	(0.17)	0.02	(0.10)	22
				Y	0.51	0.74	0.42	0.30	0.42	0.34	1
				Bl	0.74	0.79	0.51	0.61	0.58	0.78	
				Bl	0.87		0.62			0.85	75
							0.68				
109	Cibacetbraun JNH	Ciba	—	O	(0.05)	(0.45)	0.25	0.30	0.00	(0.21)	22
				Bl	(0.12)	0.70	0.42	0.61	(0.07)	(0.45)	56
				O	0.29	0.79	0.46	(0.70)	0.50	0.78	22
				Bl	0.31	0.85	0.51	0.75	0.58	0.85	98
				Ro	(0.39)		0.61	0.88		(0.92)	56
				Bl	(0.46)		(0.70)				56
				Re	0.50		0.76				35
				Y	0.51						1
				Bl	0.67						98
				V	0.78						56
				Re	(0.80)						55
				Bl	0.89						98

(continued on p. D36)

TABLE 21 (*continued*)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f] S_1	S_3	S_4	S_5	S_6	S_7	Components[g]
110	Cellitonechtbraun BG	BASF	—	O	(0.05)	(0.45)	(0.35)	(0.45)	0.05	0.45	22
				O	0.29	0.70	0.42	0.61	0.12	0.85	22
				Bl	0.31	0.75	0.46	0.70	0.58		98
				Re	0.45	0.79	0.56		0.88		
				Bl	0.67		0.62				98
				Bl	(0.76)		0.72				
				Bl	(0.89)						98
111	Delfacetbraun GD	Delft	—	Bl	(0.03)	(0.45)	0.42	0.38	0.15	(0.53)	97
				V-Bl	(0.17)	0.51		0.61	(0.57)	(0.77)	97
				Bl	(0.31)				0.83	0.89	97
				Re	0.33				0.89		
				Re	(0.50)						35
				Y	(0.61)						
				V	(0.78)						56
112	Cibacetbraun DNH	Ciba	—	Bl	(0.08)	0.23	s	0.00	0.09	0.43	94
				Bl	(0.18)	0.40	0.28	0.06	0.50	0.78	
				Bl	0.43	0.60	(0.38)	(0.20)		(0.92)	94
				Re	0.50	0.67	0.50	0.30			35
				Y	0.51	0.79	0.51	0.42			1
						0.85		(0.61)			
								0.75			
113	Setacylbraun RN	Geigy	—	Br	(0.04)	0.77	0.35	s	0.01	s	
	Setacylbraun BN	Geigy		Ro	(0.09)	0.79	0.50	0.72	0.03	(0.30)	
				O	0.22		0.63	0.81	0.22	(0.79)	
				Re	0.33			(0.89)	0.41	0.83	
				V	(0.43)				0.68	0.90	
				Bl	0.88						
114	Cibacetbraun 3 R	Ciba	—	Y	(0.05)	(0.65)	0.25	0.00	s	0.68	
				Br	0.25	0.75	0.35	0.15			
								0.24			
115	Palanilbraun 5 RN	BASF	—	Re-Br	0.26	0.75	0.31	0.38	s	s	
	Cellitonechtbraun 5 RN	BASF						(0.61)			
116	Setacylbraun 2 GR	Geigy	—	Br	0.11	(0.10)	0.22	0.29	s	0.90	
	Setacylbraun P-2 GR	Geigy				0.53		0.42	(0.85)	s	
	Krasno-koritschnevy	SSSR				(0.60)					

117	Cellitonechtbraun 5 R	BASF	11 190	Re-Br	0.10	0.48 0.63	(0.08) 0.18 (0.50)	0.28 (0.40) (0.58)	0.76 (0.94)	0.88

Structure: O_2N–C₆H₂(Cl)(Cl)–N=N–C₆H₃(CH_3)–N(CH_3)(CH_2CH_2OH) (·Cl, H_3C)

118	Cellitazol GG	BASF	11 220	V Br-Gr	(0.62) 0.96	0.88	—	0.00 0.82	0.00	0.02

Structure: O_2N–C₆H₃(OCH_3)–N=N–C₆H₃(H_3C)–$N(CH_2CH_2OH)_2$

TABLE 22

R_F VALUES OF GREEN DYES

(For notes see p. D10)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f] S_1	R_F[f] S_3	R_F[f] S_6	Components[g]
119	Artisilblaugrün GP	Sandoz	62 500	Bl	(0.56)	(0.66)	0.01	
	Cibacettürkisblau G	Ciba		Bl	0.67	(0.73)	—	
				Gr-Bl	0.77	0.80		
				Bl	0.85			
				Bl	0.90			
				Bl	0.97			
119a	+ Perlitonblaugrün B	BASF	—	Bl	(0.56)	—	0.01	
	Cellitonechtblaugrün B	BASF		Bl	0.24		0.08	
				Bl	0.04		(0.51)	
				Ro	0.09			
				Bl	0.19			
				Bl	0.67			
				Gr-Bl	0.77			
				Bl	0.85			
				Bl	0.90			
				Bl	0.97			
119b	+ Serisol Fast Blue Green BW	YDC	—	Bl	0.24	(0.66)	0.01	
				Bl	(0.56)	(0.73)	(0.08)	
				Bl	0.67	0.80		
				Gr-Bl	0.77	(0.85)		
				Bl	0.85			
				Bl	0.90			
119c•	Duranol Blue Green B	ICI	—	Bl	0.24	(0.66)	0.01	
				Bl	(0.56)	(0.73)	(0.08)	
				Bl	0.67	0.80		
				Gr-Bl	0.77	(0.85)		
				Bl	0.90			
				Bl	0.93			

Structure:

OH O $NHCH_2CH_2OH$

OH O $NHCH_2CH_2OH$

120	Setacylblaugrün BSN	Geigy	62 500	Gr	0.10	0.29	s	
				Bl	0.12	(0.34)	(0.89)	
				Bl	0.27	0.76		
				Gr	0.71	0.80		
				Bl	0.88	(0.88)		

Structure: OH O NHCH$_2$CH$_2$OH / OH O NHCH$_2$CH$_2$OH (anthraquinone ring structure)

121	Cibacetgrün 5 G	Ciba	—	Y	0.51	0.73	0.01	1
				Bl	(0.67)	0.80	0.25	119
				Gr-Bl	0.77			119
121a	Serisolbrillantgrün BN	YDC	—	Y	0.51	0.48	0.01	1
				Bl	(0.67)	0.73	(0.08)	119
				Y	0.29	0.77	0.25	
				Gr-Bl	0.77	0.80	0.92	119
121b	Cellitonbrillantgrün 3 G	BASF	—	Gr	(0.14)	0.35	0.01	
				Y	0.29	(0.48)	(0.05)	
				Y	0.51	0.66	(0.08)	1
				Bl	(0.56)	0.73	0.18	119
				Bl	(0.67)	0.77	0.25	119
				Gr-Bl	0.77	0.80		119
121c	Cellitonechtgrün FFG	BASF	—	Y	0.11	0.35	0.01	
				Bl	(0.25)	0.66	(0.08)	
				Y	0.51	0.73	0.25	1
				Bl	(0.56)	0.77	0.92	119
				Bl	(0.67)	0.80		119
				Gr-Bl	0.77			119
121d	Samarongrün 3 G	Hoechst	—	Y	0.29	0.48	0.01	
				Bl	(0.67)	0.66	0.18	119
				Gr-Bl	0.77	0.73		119
						0.80		

(continued on p. D40)

TABLE 22 (*continued*)

No.[a]	Dye[b]	Manufacturer[c]	C.I. No.[d]	Colour[e]	R_F[f]			Components[g]
					S_1	S_3	S_6	
122	Novalonechtgrün G	Geigy	—	O	0.05	—	—	22
				Bl	0.27			
				O	(0.29)			22
				Bl	0.41			
				Y	0.51			1
				Bl	0.52			
122a	Cibacet Dark Green B	Ciba	—	Re	0.24	0.69	s	
				Bl	0.27	0.76	0.25	
				Bl	0.41	0.80	0.35	
				Y	0.51		0.61	1
				Bl	0.52			
123	Cellitonechtdunkelgrün B	BASF	—	Y	0.09	0.42	0.00	
				Br-Re	(0.15)	0.48	0.06	100
				Bl	0.31	(0.58)	0.25	98
				Y	0.51	0.73	(0.52)	1
				Bl	0.67	0.77	0.88	98
				Bl	0.89	(0.80)		98
123a	Serisol Fast Green BTL	YDC	—	Y	0.06	—	—	
				V	(0.17)			
				Y	0.22			
				Bl	0.31			98
				Bl	0.67			98
				Bl	(0.85)			
				Bl	0.89	(0.05)		98
124	Cellitonechtgrün 3 BS	BASF	—	Bl	0.05	0.09	0.01	
				Bl	0.45	(0.21)	s	
						0.79		
125	Setacylbrillantgrün 2 G	Geigy	—	Gr	0.96	0.00	0.01	
				Gr	1.00	s		
126	Cellitonechtgrün 5 B	BASF	61 540	Gr	0.04	0.00	s	
				Gr	0.10	(0.05)	0.54	
				V	0.19	(0.34)		

				Gr	0.21	(0.65)		
				Gr	0.40	(0.73)		
				Gr	0.55	0.82		
				Gr	0.32			

Structure: O, $NHCH_3$, O, NH–, $–NHCH_2CH_2OH$

127	Artisilgrün BLP	Sandoz	—	Y	0.16	(0.01)	0.01	
				Y	0.34	0.64	0.08	
				Bl	0.87	0.76		75
						(0.85)		
128	Cellitonechtgrün 3 B	BASF	56 060	Bl	0.04	—	0.01	
	Seleny 24	SSSR		Gr	0.48		s	

Structure: NH, O, $–CH_2O–$, –N

TABLE 23

R_F VALUES OF BLACK DYES

(For notes see p. D10)

No.[a]	Dye[b]	Manufacturer[c]	Colour[e]	R_F[f] S_1	S_3	S_5	S_6	S_7	Components[g]
129	Cibacetschwarz B	Ciba	Gr	(0.05)	0.11	0.05	0.00	0.00	
			Bl	0.09	(0.22)	0.15	0.09	0.57	
			Bl	0.16	0.29	0.35	0.12	0.70	
			O-Br	0.20	0.54	0.44	0.18	0.91	28
			Y	0.35	0.62		0.24		
			Bl	0.45	0.64		(0.74)		
			Bl	(0.55)	0.70		(0.88)		
					0.75				
130	Setacylschwarz 2 GA	Geigy	Bl	(0.04)	0.25	0.11	0.00	0.21	
	Setacylschwarz BTV	Geigy	Gr	0.10	0.34	0.16	0.11	0.39	120
	Setacylschwarz GTA	Geigy	Bl	0.12	0.43	0.33	0.24	0.62	120
			O-Br	0.20	0.61	0.44	0.40	0.75	28
			Bl	0.27	0.76	0.53	0.71	0.81	120
			Re	0.42	0.79	0.67	0.81	0.85	48
			Y	(0.55)	0.70	0.72	0.91	0.91	48
			Gr	0.71		0.62			120
			Bl	(0.88)					120
130a	Artisilschwarz GSPN	Sandoz	Bl	(0.04)	0.25	0.11	0.00	0.05	
			Gr	0.10	0.43	0.16	0.11	0.21	120
			Bl	0.12	0.49	0.33	0.24	0.62	120
			Y	0.19	0.61	0.44	0.40	0.75	
			Bl	0.27	0.69	0.53	0.87	0.85	120
			Re	0.42	0.76	0.62		0.91	48
			Y	(0.55)	0.79				48
			Gr	0.71					120
			Bl	(0.88)					120
131	Setacylschwarz BGA	Geigy	Bl	0.04	—	0.00	0.00	0.21	
			Bl	0.12		0.33	0.05	0.29	120
			Y	0.19		0.44	0.08	(0.65)	
			Bl	0.27		0.53	0.15	0.81	120
			Ro	0.40		0.79	0.40	0.90	45
			Bl	(0.90)			0.87		120
132	Setacylschwarz BSN	Geigy	Re	0.36	0.67	s	0.00	0.02	46

	Artisilschwarz BSN	Sandoz	Y	0.55	0.71	0.22	0.32	0.09	1
			Bl	0.89	0.75	0.27	0.39	(0.26)	99
					0.79	0.77		0.76	
					0.82			0.82	
133	Cibacetschwarz TNH	Ciba	Bl	0.04	—	0.00	0.00	0.02	92
			Bl	0.09		0.05	0.10	0.10	92
			Gr	(0.13)		0.22	0.20	0.64	92
			V	0.17		0.25	0.25	0.70	92
			Re	0.24		0.33	(0.36)	0.75	
			O	0.29		0.43	(0.54)	0.85	22
			Bl	0.46		0.56	(0.88)	0.92	92
			Y	0.51					1
			Bl	(0.69)					
			Gr	0.71					120
134	Perlitonschwarz B	BASF	Bl	0.24	0.68	s	0.00	s	
			O	0.29	0.72	0.42	0.04	0.26	74; 22
			Re	0.42	0.78	0.58	(0.10)	0.35	
			Bl	(0.69)	0.85	0.44	0.54	(0.65)	
			V	0.72			(0.15)	0.85	74
			Bl	(0.86)					75; 74
134a	Cellitonechtschwarz B	BASF	O	0.29	0.68	s	0.00	s	74; 22
			Re	0.42	0.72	0.42	0.02	0.26	
			Bl	(0.69)	0.78	0.58	0.10	0.35	
			V	0.72	0.85		0.54	(0.65)	74
			Bl	(0.86)			0.04	0.85	75; 74
135	Cibacetschwarz GD	Ciba	O	(0.05)	—	s	s	s	22
			Bl	(0.19)		0.35	0.54	(0.26)	
			O	0.29		0.58	0.86	(0.35)	22
			Gr	0.42		0.72		0.50	
			Re	(0.51)				0.85	
			V	0.78				(0.92)	56
			Bl	0.87					75
136	Cellitonechtschwarz BTNV	BASF	O	(0.05)	0.42	0.27	s	s	22
			Re	0.15	0.57	0.58	0.54	0.50	
			O	0.29	0.62		0.58	0.85	22
			V	0.31	0.68		0.62	0.88	57
			Ro	0.39	0.71		0.88	0.92	57
			Bl	0.60					57
			V	0.78					57
			Bl	0.89					99

(continued on p. D44)

TABLE 23 (*continued*)

No.[a]	Dye[b]	Manufacturer[c]	Colour[e]	R_F[f] S_1	S_3	S_5	S_6	S_7	Components[g]
136a	Cellitonechtschwarz BTN	BASF	O	(0.05)	0.42	0.27	s	s	22
			Re	0.15	0.57	0.54	0.54	0.50	
			O	0.29	0.62	0.58	0.58	0.85	22
			V	0.31	0.68		0.62	0.88	57
			Ro	0.39	0.71		0.88	0.92	57
			Bl	0.60					57
			Bl	(0.67)					119
			V	0.78					57
			Bl	0.89					99
137	Cellitonechtschwarz GN	BASF	Gr	(0.13)	0.42	0.15	0.00	s	
			Bl	(0.24)	0.58	0.27	0.05	0.50	
			O	(0.29)	0.62	0.54	0.10	0.55	22
			Br-Re	0.17	0.71		0.58	0.85	
			Bl	(0.31)	0.76		0.64	0.88	57
			Ro	(0.39)			0.88	0.92	57
			Bl	0.60					57
			Gr	0.74					120
			V	0.78					57
			Bl	(0.89)					99
137a	Cellitonechtschwarz 3 G	BASF	Br-Re	0.17	—	0.15	—	—	
			Bl	(0.24)		0.27			
			Bl	0.29		0.54			
			Bl	(0.31)					57
			Ro	(0.39)					57
			Bl	0.60					57
			Gr	0.74					120
			V	0.78					57
			Bl	(0.89)					99
138	Cibacetschwarz 711	Ciba	Re-Br	0.05	0.58	0.06	0.10	(0.05)	
	Cibacetschwarz TW	Ciba	Re	0	0.67	0.34	0.15	(0.26)	
			Re-Br	0.17	0.71	0.42	0.24	0.50	
			Bl	0.26	0.76	0.44	0.70	0.60	91
			Bl	0.46		0.50	0.76	0.71	91
			Bl	0.55				0.90	91
			Re	(0.66)					
			V	0.78					56

			[illegible]			[illegible]		[illegible]	
			Bl	[illegible]	[illegible]	0.58	0.24	0.56	
			O	0.29	0.68	(0.65)	0.54	0.60	22
			Bl	0.31	0.71		0.67	0.70	98
			Re	0.38	0.76		0.88	0.85	
			Bl	0.46				0.92	92
			Gr	0.55					
			Bl	0.67					98
			V	0.78					56
			Bl	0.89					98
139a	Artisilschwarz RTNN	Sandoz	O	(0.05)	0.36	0.22	s	0.04	22
			Re	0.16	0.42	0.25	(0.15)	0.50	
			Bl	0.25	0.68	0.35	0.24	0.56	
			O	0.29	0.71	0.50	(0.36)	0.70	22
			Bl	0.31	0.74	(0.58)	0.49	0.76	98
			Bl	0.46	0.76	(0.65)	0.54	0.85	92
			Gr	0.55	0.61	0.72	0.80	0.92	
			Bl	0.67			0.88		98
			V	(0.78)			(0.95)		56
			Bl	(0.89)					98
140	Serisol Direct Black GT	YDC	O	(0.05)	(0.42)	—	0.00	—	22
			Re	0.15	0.57		0.04		
			O	0.29	0.64		0.07		22
			Bl	0.31	0.71		(0.10)		98
			Bl	0.67			(0.60)		98
			Bl	0.89			0.70		98
							(0.88)		
140a	Serisol Direct Black GR	YDC	O	(0.05)	0.42	—	0.00	—	22
			O	0.29	0.64		0.10		22
			Bl	0.31	0.71		0.20		98
			Ro	0.39			0.70		56
			Bl	0.67			0.88		98
			V	0.78					56

TABLE 24

R_F VALUES OF GRAY DYES

(For notes see p. D10)

No.[a]	Dye[b]	Manufacturer[c]	Colour[e]	R_F[f]			Components[g]
				S_1	S_6	S_7	
141	Perlitongrau N	BASF	Bl	0.20	0.00	0.00	82
			Bl	(0.26)	0.05	(0.04)	82
			Re	0.50	0.42	(0.18)	35
			Y	0.51	0.85	0.38	1
			Bl	(0.52)		0.48	82
			Gr-Bl	0.62		0.84	82
			V-Bl	0.67		0.96	82
			Gr-Bl	0.74			82
142	Cibacetgrau NH	Ciba	Bl	0.31	0.00	0.00	98; 97
			Re	0.50	0.05	0.03	35
			Y	0.51	0.11	0.48	1
			V-Bl	0.67	0.42	0.61	98
			Bl	(0.88)	0.90	0.84	98; 99
143	Novalongrau BRL	Geigy	Br-Y	(0.21)	0.00	0.15	
			Bl	0.31	0.15	0.73	97
			Re	0.42	0.89	0.96	48
			Y	0.55	0.91		48
			Gy-Bl	(0.98)			
143a	Novalonechtgrau RB	Geigy	Br-Y	(0.21)	0.00	0.00	
			Bl	0.31	0.15	0.73	97
			Re	0.42	0.60	0.89	48
			Y	0.55	0.89	0.96	48
			Bl	0.58	0.91		
			Gy-Bl	(0.98)			
144	Novalongrau BRN	Geigy	Bl	(0.26)	0.00	0.00	93
			Bl	(0.37)	0.02	(0.26)	93
			Re	0.42	0.15	0.38	
			Bl	0.49	0.42	(0.58)	93
			Y	0.51		0.73	
						0.84	
145	Cellitonechtgrau BG	BASF	Y	0.09	0.00	0.00	3
			Bl	(0.18)	0.11	0.27	
			Re	(0.23)	0.82	0.58	

			...	0.32		0.94	
			Bl	0.76			
			Bl	(0.91)			
145a	Cellitonechtgrau GGU	BASF	O	(0.05)	0.00	0.00	22
			Y	0.09	0.01	(0.06)	3
			Bl	(0.18)	0.13	0.24	
			Re	(0.25)	0.24	0.58	
			O	0.29	0.61	(0.76)	22
			Bl	0.76	0.82	0.89	
			V	0.78		0.94	56
			Bl	(0.91)			
146	Artisilgrau BLP	Sandoz	O	(0.05)	0.00	0.00	22
			Bl	(0.19)	0.06	0.10	
			Re	0.23	0.56	(0.50)	
			O	0.29	0.61	0.90	22
			Bl	0.31	0.91	0.96	97
			Y	(0.83)			
			Bl	0.87			75
147	Setacylgrau BS	Geigy	Bl	(0.02)	0.00	0.00	
			Gr	(0.10)	0.38	0.24	120
			Bl	(0.12)	(0.42)	0.83	120
			Re	0.23	0.55	0.91	
			Bl	0.27			120
			Y	0.51			1
			Gr	0.71			120
			Bl	0.88			120

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