JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

VOL. 14 1964

ELSEVIER PUBLISHING COMPANY

AMSTERDAM

ELSEVIER PUBLISHING COMPANY

PRINTED IN THE NETHERLANDS BY
DRUKKERIJ MEIJER N.V., WORMERVEER

CHROMATOGRAPHIC STUDIES OF SILICON COMPOUNDS

I. USE OF A FLAME IONISATION GAUGE IN DETERMINATION OF RETENTION DATA

F. H. POLLARD, G. NICKLESS AND P. C. UDEN

Department of Chemistry, The University, Bristol (Great Britain)

(Received July 24th, 1963)

INTRODUCTION

As part of a project involving accurate retention data measurements for volatile organo-silicon compounds, and the gas chromatographic study of their reactions, the possibility of using a flame ionisation detector with associated high sensitivity has been studied.

Flame ionisation detectors of various design[1,2] have been described, their theoretical operation has been discussed[3], but little reference to the detection of either π or σ-bonded organo-metallic compounds has been made.

In this work the detector employed was similar in design to that described by the Associated Octel Co.[4], and appropriate experimental conditions enabled specific retention data[5] to be obtained for series of Group IV alkyls, hydrocarbons and chloro compounds.

EXPERIMENTAL

Detector (see Fig. 1)

The column consisted of 6 ft. copper U-tubing of $^1/_4$ in. O.D., set vertically in a vapour jacket. Samples were injected by means of Hamilton μl syringes or 1 ml gas-tight syringes into a T joint compression fitting at the column inlet. The detector flame chamber was mounted horizontally at the end of the column, on a brass plate (6 cm^2) over which a screw cover could be fitted. Through the centre of the bottom of the plate was silver-soldered a compression fitting into which the end of the column passed. A piece of glass tubing (3 mm O.D.), with its top 5 mm conically ground, over which a Record hypodermic needle could be fitted, was sealed into this fitting to give insulation from chamber and column. A size 16 needle was cut square to form the jet for the flame. The burner tip was located between two 4 mm^2 platinum electrodes placed parallel, and 5 mm apart (found to give optimum sensitivity), supported on K.L.G. type lead-through insulators soldered into the chamber base. B.O.C. white spot N_2 carrier gas was used, high purity H_2 passed in below the plate (at about 30 ml/min controlled through a needle valve by a capillary manometer), and air for combustion entered the chamber radially at the rate of approximately 400 ml/min, air turbulence being minimised by a $^1/_2$ in. layer of glass beads in the chamber base.

The ionisation current passing through the flame under the influence of a polarising potential of 70 V was fed via an amplifier to a potentiometric recorder.

The amplifier used[6] was of a simple impedence matching type, having a sensitivity control giving full-scale deflections of 10 mV for ionisation currents in the range 10^{-6}–10^{-9} A. Care was necessary in earthing of the electrode leads and also amplifier casing as inadequate earthing (resistance less than 100 MΩ) could cut out signals completely.

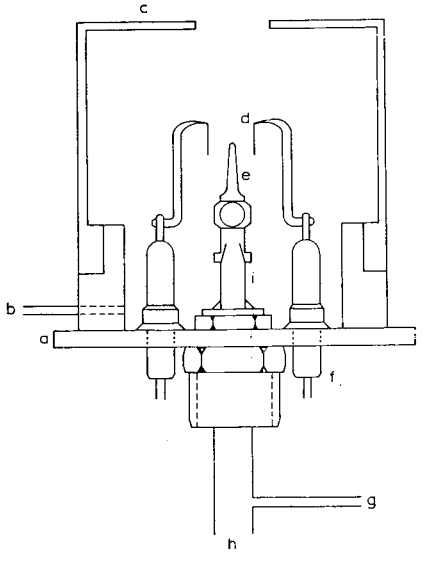
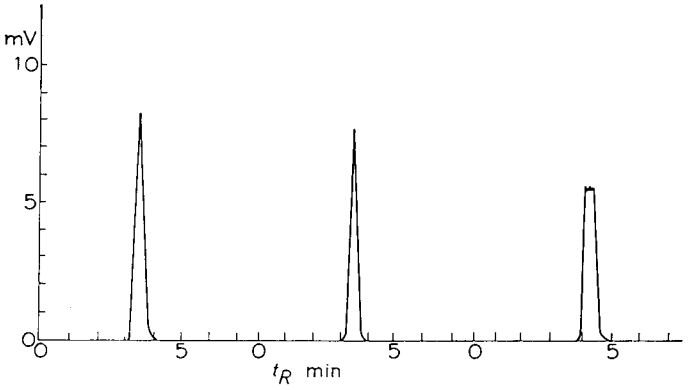


Fig. 1. Flame ionization gauge detector a = brass-base plate; b = $^1/_8$ in. O.D. copper tube for compressed airflow; c = brass cover plate; d = platinum electrode plates; e = jet (No. 16 hypodermic needle); f = ceramic lead-through connectors; g = hydrogen line; h = nitrogen from column; i = glass tubing.

Chromatography of Group IV alkyls

For all liquid samples (even samples of a fraction of 1 μl) saturation of some kind occurred in the detector, as soon as the ionisation current exceeded approximately $5 \cdot 10^{-5}$ mA. The type of trace found is seen in Fig. 2 for samples Me_3CEt, Me_3CCl, and Me_3SiEt; the flat cut off for the silicon compound being noted. No sensitivity change could remove this fault which was assumed to be due to actual ionisation characteristics in the flame. The assumption of the absence of an actual coating on the electrodes being responsible for this saturation seems justified, since organic compounds chromatographed immediately afterwards showed no change in response.

This difficulty can be overcome by two methods involving decrease in sample size: (1) injection of small samples as vapour in conjunction with the use of the detector at maximum sensitivity, and (2) employment of a bleed-off valve (stream-splitter) between injection seal and column. The data given were obtained by the former method, although the latter has also been used in similar work. Vapour samples taken from above the liquid surface at room temperature gave normal symmetrical peaks giving consistent retention data through the various series. The sizes of vapour/air samples required depended obviously on the vapour pressures of the compounds, but 1 μl to 1 ml sample ranges for 26° to 200° (containing in the order of 10^{-10} g) gave ionization currents of 10^{-8}–10^{-9} A, *i.e.* below the saturation limit.



i) $(CH_3)_3C(C_2H_5)$ ii) $(CH_3)_3$ CCl iii) $(CH_3)_3$ $Si(C_2H_5)$

Fig. 2. Chromatographic characteristics for analogous carbon and silicon compounds. Stationary phase: 15% E 301 silicone oil, 80°; carrier gas: nitrogen; flow rate 24.7 ml/min; 1 μl liquid sample; F.S.D. $\simeq 10^{-7}$ A.

Preparation of alkyl silanes

These were prepared by Grignard reactions of the appropriate alkyl halide with chlorosilanes in di-ethyl ether or di-*n*-butyl ether, having 20% excess of Grignard reagent, followed by fractionating employing purity checking by GLC. Purity was estimated in all cases as $> 98\%$[7].

The course of the fractional distillation of the multi-component system which is always encountered in Grignard reactions of this type, is commonly followed by noting the changes in boiling point and refractive index changes. Such techniques are not completely reliable in such cases in which the boiling points are very close or the differences in refractive indices are small. Often recourse can be made to changes in the infra-red spectra of the fractions, but reliable data on the mixed tetraalkylsilanes are not available. Gas chromatography offers an important and indispensable tool for following fractional distillations of such σ-bonded organo-metallic compounds. Analysis of each fraction as the distillation proceeds, enables a more judicious choice (to be made) in effecting the best separation of components, and therefore affords a maximum amount of each component.

Fig. 3 gives an example of the use of gas chromatography in the isolation and purification of trimethylethylsilane.

(*a*) *Fractionation.* See the following scheme.

No.	*Attenuation*	*Boiling point range (°C)*	*Components*
1	50	→ 35	I, II, IV
2	50	35–58	I, II, III, IV
3	50	56–60	I, II, III, IV
4	100	57–63	–, II, III, IV
5	100	63	– – III, IV
6	100	63–65	– – III, IV
7	100	63–67	– – III, IV
8	100	Residue	– – – IV, V

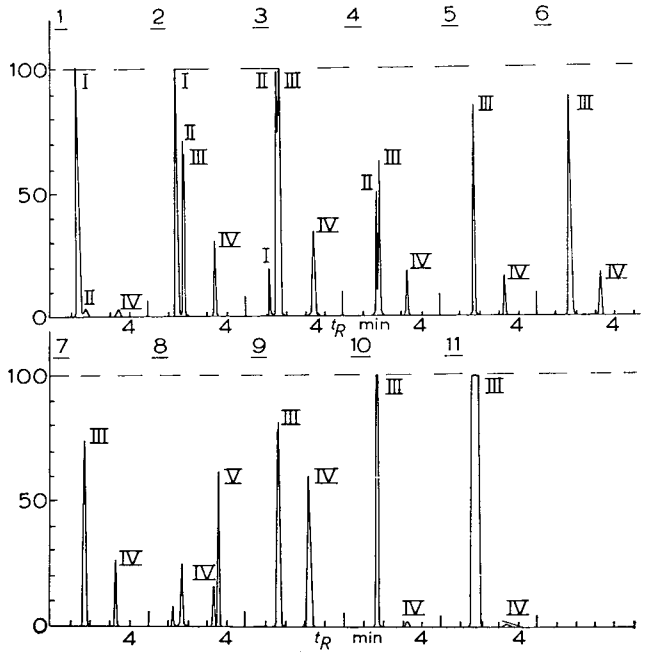


Fig. 3. Gas–liquid chromatography analysis in the preparation of $(CH_3)_3SiC_2H_5$. Column: 2 m of 15% E 301 silicone oil on 36–60 celite (treated), 80°; detector: gas density balance; carrier gas: nitrogen, flow rate 35 ml/min; 4 μl samples injected. I = diethyl ether; II = trimethylchlorosilane; III = trimethylethylsilane; IV = hexamethyldisiloxane.

(*b*) *Purification.* The fractions containing yields of III were hydrolysed with water to convert III to IV.

9	(originally fraction 3)	100	—	III, IV

Finally, to remove IV, the fractions were shaken with successive portions of concentrated sulphuric acid.

10	(originally fraction 9)	100	—	III, IV

Fractions 2–7 were subjected to the water followed by acid treatment, and the fractions combined, and redistilled to produce fraction 11.

11		5	63.5°	III, IV

From the detector response, the purity of the Me_3SiEt was taken as at least 99.8%.

Preparation of tin alkyls[8]

$SnMe_4$ and $SnEt_4$ were prepared by Grignard reactions from $SnCl_4$, while the intermediate alkyls were prepared by bromination in equivalent proportions to give Me_3SnBr, Me_2SnBr_2 and $MeSnBr_3$ followed by the appropriate Grignard reactions and fractional distillation. $GeMe_4$ was made similarly.

Chlorosilanes, hexamethyldisiloxane, normal alkanes, lead tetraethyl and chlorinated hydrocarbons were obtained commercially.

RESULTS

The compounds chromatographed included hydrocarbons and chlorinated compounds to provide some comparison for specific retention data, namely:

$SiMe_4$	$SnMe_4$	$GeMe_4$	Me_3SiCl	Me_3CCl	C_6H_6
Me_3SiEt	Me_3SnEt		Me_2SiCl_2	Me_2CCl_2	n-C_5H_{12}–n-C_9H_{20}
Me_2SiEt_2	Me_2SnEt_2		$MeSiCl_3$	$HCCl_3$	
$MeSiEt_3$	$MeSnEt_3$		$SiCl_4$	CCl_4	
$SiEt_4$	$SnEt_4$	$PbEt_4$			

Me_3SiPr-n, Me_3SiBu-n, Et_3SiH, $Me_3SiOSiMe_3$

With the apparatus described as above, consistent results for vapour samples were obtained for all except the chlorosilanes.

Trimethylchlorosilane showed almost complete hydrolysis to hexamethyldisiloxane, while the di- to tetrachloro compounds gave no response. This was assumed to

TABLE I

SPECIFIC RETENTION VOLUMES V_g (ml) ON 15% E 301 SILICONE OIL

Compound	*80°*	*100°*	*110.5°*	*140°*
$SiMe_4$	11.82 ± 0.15	7.65 ± 0.07	6.31 ± 0.07	3.09 ± 0.03
Me_3SiEt	31.4 ± 0.2	18.45 ± 0.06	13.77 ± 0.2	7.51 ± 0.05
Me_2SiEt_2	79.2 ± 0.5	43.2 ± 0.2	32.0 ± 0.7	16.4 ± 0.2
$MeSiEt_3$	190.9 ± 0.9	98.7 ± 0.7	68.3 ± 1.8	34.1 ± 0.1
$SiEt_4$	446.8 ± 2.7	216.8 ± 3.0	147.7 ± 2.9	69.3 ± 0.4
Me_3SiPr-n	61.6 ± 0.6	34.3 ± 0.4	24.9 ± 0.1	13.33 ± 0.2
Me_3SiBu-n	129.3 ± 2.0	68.0 ± 0.2	47.7 ± 0.2	24.3 ± 0.1
Et_3SiH	118.8 ± 0.4	64.6 ± 1.1	44.8 ± 0.6	23.2 ± 0.1
$Me_3SiOSiMe_3$	74.4 ± 0.5	40.1 ± 0.1	28.4 ± 0.4	14.7 ± 0.05
$GeMe_4$	20.1 ± 0.05	12.37 ± 0.12	9.29 ± 0.1	—
$SnMe_4$	50.1 ± 0.3	28.4 ± 0.1	21.0 ± 0.4	11.2 ± 0.1
Me_3SnEt	116.6 ± 1.0	62.3 ± 0.3	43.9 ± 0.3	22.5 ± 0.3
Me_2SnEt_2	264.3 ± 3.2	131.2 ± 1.0	91.3 ± 1.2	44.0 ± 0.4
$MeSnEt_3$	577.0 ± 9.1	270.5 ± 1.8	181.9 ± 0.9	82.7 ± 1.0
$SnEt_4$	122.4 ± 10.0	546.4 ± 7.0	355.0 ± 3.3	153.9 ± 2.4
$PbEt_4$	—	1171.5 ± 9.0	713.3 ± 3.3	293.1 ± 5.0
Me_3CEt	23.4 ± 0.3	14.3 ± 0.1	10.81 ± 0.15	5.98 ± 0.03
Me_3CCl	24.1 ± 0.4	14.8 ± 0.2	11.2 ± 0.25	6.36 ± 0.05
Me_2CCl_2	40.6 ± 0.3	24.0 ± 0.2	17.8 ± 0.2	9.93 ± 0.09
$HCCl_3$	40.0 ± 0.3	23.5 ± 0.1	17.4 ± 0.3	9.65 ± 0.1
CCl_4	63.5 ± 0.2	36.2 ± 0.3	26.4 ± 0.5	14.5 ± 0.11
C_6H_6	60.6 ± 0.8	34.8 ± 0.05	25.4 ± 0.4	14.2 ± 0.1
n-C_5H_{12}	17.1 ± 0.2	10.76 ± 0.08	8.17 ± 0.15	4.62 ± 0.02
n-C_6H_{14}	38.5 ± 0.2	22.5 ± 0.01	16.8 ± 0.2	8.65 ± 0.05
n-C_7H_{16}	84.8 ± 0.8	46.2 ± 0.04	33.1 ± 0.6	16.9 ± 0.08
n-C_8H_{18}	184.7 ± 1.8	94.2 ± 1.1	65.2 ± 0.8	31.8 ± 0.1
n-C_9H_{20}	400.7 ± 6.9	188.4 ± 2.0	129.1 ± 2.9	58.8 ± 0.7

be due to complete hydrolysis of the vapour and not detector or column characteristics, since these compounds have since been chromatographed with appropriate precautions to minimise hydrolysis both with this detector using the stream splitting system for liquid samples, and also on a katharometer.

Retention data are recorded on 15 % silicone oil E 301, (mol. wt. 700,000) on Celite treated[9] by dry-sieving to mesh 36–60, washing with conc. HCl, methanol, and distilled water, followed by drying at 300°; phases of this type were used in the chromatography of chlorosilanes[10].

The values of specific retention volumes V_g given in Table I are the mean values at each temperature of determinations at inlet/outlet pressure ratios of 1.2, 1.4 and 1.6.

Errors given on this mean are in almost all cases less than $\pm$ 2%; this being smaller than that which may be expected from experimental error.

DISCUSSION

Vapour samples taken as described are useful in the measurement of accurate specific retention data, since they give an approximation to ideal chromatography, *i.e.* sample size approaching zero, and injection as a plug of vapour. However, for quantitative sensitivity measurement the disadvantages are: (i) uncertainty as to exact quantities, and (ii) diminution of sample size taken for higher boiling compounds.

Among items of interest in the range of compounds detected are: (a) the ability to detect chloro compounds, *e.g.* CCl_4 with relatively little loss of sensitivity, (b) the response of the detector to silicon compounds of similar boiling point to analogous carbon compounds, is approximately 20 % greater, and (c) evidence for the formation of ions in the flame deriving from the case of lead tetraethyl. Here, after injection of a liquid sample, a red deposit was formed on the negative electrode, thus indicating the formation of positive lead ions in the flame.

GRAPHICAL TREATMENT OF RETENTION DATA

A plot (Fig. 4) of $\log_{10} V_g$ against column temperature for members of the various series shows good straight lines at temperatures of 80°, 100° and 110.6°, but marked discontinuity at 140°. Since the relative displacement is similar for all compounds, and a straight line extrapolation would indicate a column temperature some 8–10° lower than that used, there would seem to be some discontinuity in stationary phase retention characteristics between 110.6° and 140°. (The only exception being in the case of the very small retention volume obtaining for silicon tetramethyl at 140°). This phenomenon which is being further investigated, may be compared with a similar discontinuity observed with Apiezon L between 58° and 80°[11].

Figs. 5 and 6 show plots of log V_g and number of carbon atoms for the silicon and tin tetraalkyls, and also the normal paraffins at column temperatures of 80° and 140° respectively. (The intermediate temperature graphs show parallel characteristics.)

In comparison with the paraffins which show a close approximation to a linear relationship, both mixed methylethyltetraalkyl series show a slight but consistent curvature, giving rather higher $\log_{109} V_g$ values for the asymmetrical members than

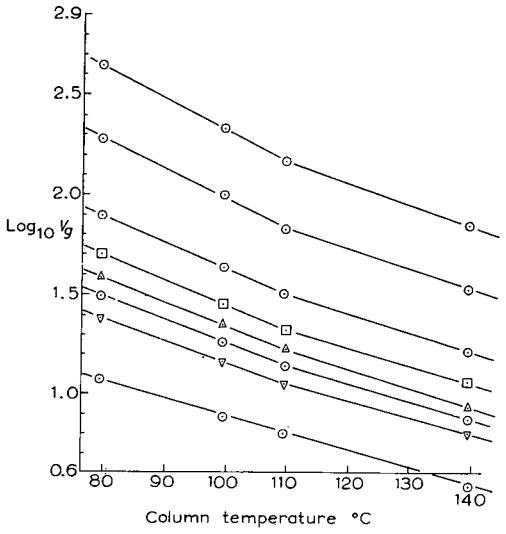


Fig. 4. Plot of $\log_{10} V_g$ against column temperature. ⊙ = tetraalkylsilanes, $SiMe_4$–$SiEt_4$; ⊡ = tetramethylstannane; △ = *n*-hexane; ▽ = *tert.*-butyl chloride.

might be predicted from numbers of carbon atoms only. This indicates some degree of selective retention of the asymmetrical over the symmetrical members, although such an effect should become more pronounced for the mixed methyl-*n*-propyl and methyl-*n*-butyl series at present being investigated. The straight chain trimethylalkyl series, however, appears to show curvature in the opposite direction, perhaps indicating some selective retention as the chain lengthens.

Fig. 7 shows a plot of $\log_{10} V_g$ against boiling point giving closely similar plots for

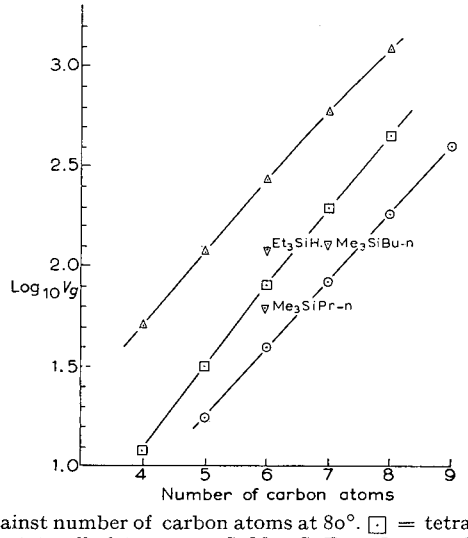


Fig. 5. Plot of $\log_{10} V_g$ against number of carbon atoms at 80°. ⊡ = tetraalkylsilanes, $SiMe_4$–$SiEt_4$; △ = tetraalkylstannanes, $SnMe_4$–$SnEt_4$; ⊙ = *n*-alkanes.

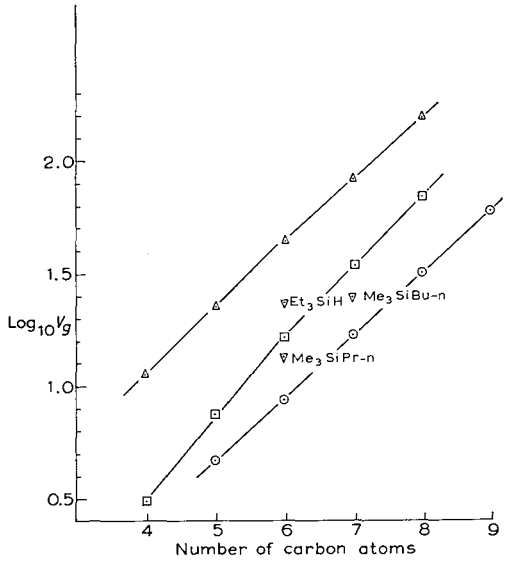


Fig. 6. Plot of $\log_{10} V_g$ against number of carbon atoms at 140°. ⊡ = tetraalkylsilanes; △ = tetraalkylstannanes; ⊙ = *n*-alkanes.

the four column temperatures. A general upward curve for each temperature is noted, giving higher retention volumes than predicted for the higher boiling compounds. A number of compounds, however, show some exception to this general trend. Trimethyl-*n*-propyl- and *n*-butylsilanes always appear somewhat below the

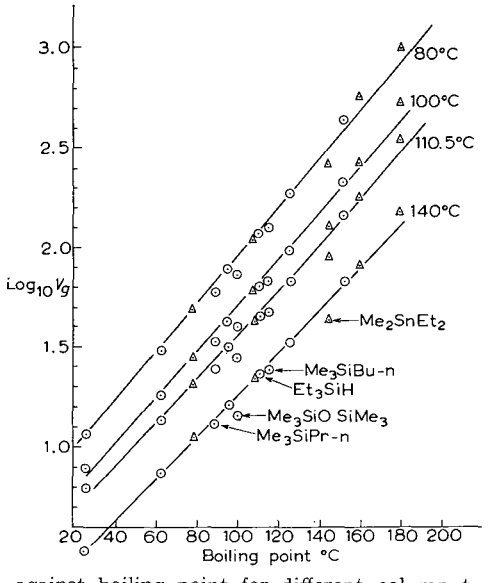


Fig. 7. Plot of $\log_{10} V_g$ against boiling point for different column temperatures. ⊙ = tetraalkylsilanes; △ = tetraalkylstannanes.

curve, but the two major exceptions are hexamethyldisiloxanes which always have a markedly lower retention volume than predicted from its boiling point (this has also been noted on tritolyl phosphate and Apiezon L phases[11]), and dimethyldiethyl tin.

This compound always appears so far below the curve that to place it on the line would mean assigning a boiling point of 138° instead of 144°. This anomalous result warrants further study since the boiling point range of the methylethyl tins, (*i.e.* 78°, 108°, 145°, 159° and 181°) shows successive differences on replacement of methyl by ethyl of 30°, 37°, 14° and 27°, in comparison with a similar series of differences in the analogous silicon series of 37°, 32°, 31° and 26°. It is seen in fact, that a boiling point of 138° assigned to dimethyldiethyl tin would make for a much closer comparison between the series.

ACTIVITY COEFFICIENT MEASUREMENTS

HARDY[12] referred to recent papers on the study of the thermodynamics of systems of involatile solvents and volatile solutes by gas chromatography, and indicated the accuracy possible in experiments having the necessary degree of control and measurement of operating parameters.

Generally, for non-polar solutes and solvents, values of activity coefficients at infinite dilution (γ°) are found to be in the range 0.1–1.0 when calculated from the equation:

$$\gamma_p^\circ = \frac{N_{\mathrm{liq}} RT}{kp_o}$$

where k is the partition coefficient obtained by dividing V°_r by the volume of the stationary phase at the column temperature, V°_r being here given as the product of the specific retention volume V_g and the weight of solvent. N_{liq} is the number of moles of liquid phase per unit volume at temperature T°K.

Thus:

$$\gamma_p^\circ = \frac{W_l \times RT}{M \times V_l} + \frac{V_g \times W_l}{V_l \times p} = \frac{RT}{V_g \times M \times p} \qquad (1)$$

where p = vapour pressure in mm Hg of solute at temperature T,
W_l = weight of liquid phase,
V_l = volume of liquid phase,
M = molecular weight of liquid phase.

Thus accurate molecular weights, vapour pressure values and specific retention data are needed for such measurements. Since, however, the molecular weight of E 301 is approximately 700,000, it would seem likely that the γ_p° values would be very small. Although, as HARDY states, accurate values cannot be obtained under these conditions, series of relative values for various series of compounds may be calculated. Although it is doubtful whether such values are strictly activity coefficients, they do represent some analogous thermodynamic property of the system and may thus be of some interest.

Thus where possible, these values have been calculated, the limiting factor being the availability of accurate vapour pressure data over the temperature range chromatographed.

Antoine equations of the type:

$$\log_{10} p = A - \frac{B}{(c + t)} \quad (t \text{ in } °C)$$

are available[13] for the *n*-paraffins, benzene, neohexane, chloroform and carbon tetrachloride, and equations of the type:

$$\log_{10} p = C - \frac{B}{T} \quad (T \text{ in } °K)$$

for tin tetramethyl and lead tetraethyl.

Tabulated values up to their boiling points[13] are available for trimethylethyl, *n*-propyl- and *n*-butylsilanes, methyltriethylsilane, tetraethylsilane, hexamethyldisiloxane and trimethylethyl tin, which give almost linear plots of $\log_{10} p$ against $1/T°K$ (Fig. 8). Interpolation of the required values of $1/T°K$ corresponding to column temperatures, gives appropriate vapour pressure values (assuming linearity to hold

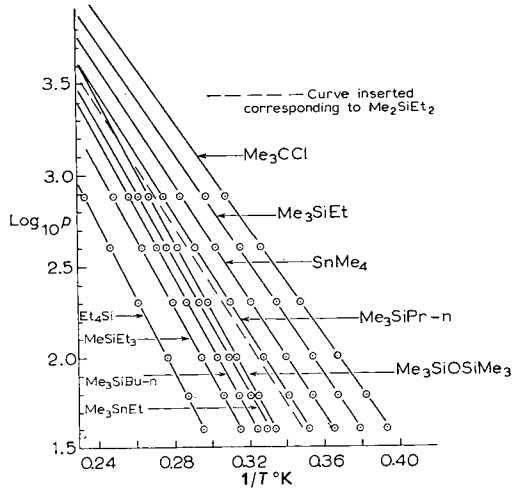


Fig. 8. Vapour pressure curves, $\log_{10} p$ (p in mm Hg) against $1/T°K$.

above the boiling point where this applies). Values for dimethyldiethylsilane were estimated by inserting its boiling point in the $\log_{10} p$ against $1/T°K$ plot, and constructing the best plot by comparison with the other members of the methylethylsilane series.

The values calculated from eqn. (1) designated by γ_N are given in Table II.

CONCLUSIONS

A number of general points may be made on the values calculated. As anticipated from eqn. (1), and the high molecular weight of the solvent, the values were of the order of 1/1000 of those found previously (*e.g.* for halogenated hydrocarbons on silicone 702, mol. wt. 530[12]). This factor is seen to be of the order of the molec-

TABLE II

VALUES OF γ_N ON 15% E 301 SILICONE OIL, GIVEN TO ONE DECIMAL PLACE (SHOWING THE SECOND AS CALCULATED)

Compound	$\gamma_N \times 10^{-4}$			
	80°	100°	110.5°	140°
$SiMe_4$	7.6 (1)	7.7 (2)	7.9 (2)	9.2 (3)
Me_3SiEt	7.7 (7)	8.2 (6)	8.7 (2)	8.8 (3)
Me_2SiEt_2	8.4 (9)	9.0 (3)	9.1 (0)	9.5 (7)
$MeSiEt_3$	9.5 (1)	9.9 (2)	10.9 (2)	11.2 (9)
$SiEt_4$	9.6 (3)	10.1 (3)	10.7 (3)	9.9 (2)
Me_3SiPr-*n*	9.0 (8)	9.7 (5)	10.4 (8)	10.7 (7)
Me_3SiBu-*n*	9.6 (9)	9.9 (9)	10.6 (0)	10.2 (5)
$Me_3SiOSiMe_3$	10.8 (1)	10.8 (5)	10.9 (8)	10.6 (1)
Me_3CEt	7.3 (9)	7.8 (6)	8.2 (8)	9.0 (0)
Me_3CCl	7.1 (0)	7.5 (4)	7.6 (8)	7.8 (3)
$HCCl_3$	5.9 (9)	6.3 (1)	6.7 (9)	6.8 (6)
CCl_4	5.8 (7)	6.2 (2)	6.7 (2)	6.7 (7)
$SnMe_4$	7.7 (8)	8.2 (3)	8.6 (8)	9.1 (8)
Me_3SnEt	8.5 (3)	8.8 (7)	9.4 (6)	9.1 (7)
$PbEt_4$	—	7.9 (1)	8.0 (0)	—
C_6H_6	6.8 (5)	7.2 (2)	7.5 (4)	7.2 (1)
n-C_5H_{12}	6.6 (7)	6.9 (9)	7.5 (3)	8.0 (2)
n-C_6H_{14}	7.6 (4)	7.9 (4)	8.4 (5)	9.2 (7)
n-C_7H_{16}	8.6 (6)	9.0 (3)	9.6 (3)	9.7 (5)
n-C_8H_{18}	9.7 (2)	10.0 (3)	10.6 (8)	10.4 (1)
n-C_9H_{20}	10.8 (0)	11.1 (8)	11.6 (0)	11.3 (0)

ular weight difference between these two silicone oils. The effect of increase in temperature produces a characteristic increase in γ_N with the exception of the 140° values, which are lower than anticipated. This break in the trend thus follows the discontinuity already noted for the log V_g-column temperature graph. A fairly regular increase is usually noted with increase in boiling point through a series, although the chlorinated hydrocarbons show lower values than expected from boiling point only. Hexamethyldisiloxane again shows some departure from the general trends, having a relatively higher value of γ_N.

ACKNOWLEDGEMENTS

The authors wish to thank D.S.I.R. for the award of Maintenance Grant to P.C.U. They also thank Unilever Ltd., and the Associated Octel Company for financial support for the provision of apparatus. Supplies of chlorosilane samples were generously given by Midland Silicones Ltd.

SUMMARY

The use of a flame ionisation detector in the chromatography of Group IV alkyls and analogous compounds is described including some discussion of unusual sensitivity characteristics in their chromatography. Specific retention data are calculated and correlated, and thermodynamic quantities similar in type to an activity coefficient at infinite dilution are calculated and discussed.

REFERENCES

1 J. HARLEY, W. NEEL AND V. PRETORIOUS, *Nature*, 181 (1958) 177.
2 I. G. MCWILLIAM AND R. A. DEWAR, *Nature*, 181 (1958) 760.
3 R. A. DEWAR, *J. Chromatog.*, 6 (1961) 312.
4 *Chromatographic Analyser for Hydrocarbon Gases*, Serial No. OP. 60/1, Associated Octel Co., London.
5 R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, London, 1960, p. 423.
6 F. R. CROPPER AND A. HEYWOOD, private communication.
7 F. H. POLLARD, G. NICKLESS AND P. C. UDEN, to be published.
8 F. H. POLLARD, G. NICKLESS AND D. J. COOKE, *J. Chromatog.*, 13 (1964) 48.
9 A. T. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 50 (1952) 679.
10 G. FRITZ AND D. KSINSIK, *Z. Anal. Chem.*, 304 (1960) 241.
11 F. H. POLLARD, G. NICKLESS AND P. C. UDEN, *J. Chromatog.*, 11 (1963) 312.
12 C. J. HARDY, *J. Chromatog.*, 2 (1959) 490.
13 T. EARL JORDAN, *Vapour Pressures of Organic Compounds*, Interscience Inc., New York, 1954.

A RECORDING CHROMATOGRAPH BASED ON JANÁK'S PRINCIPLE

G. KATEMAN AND J. A. RIJKS

Laboratorium Stikstofbindingsbedrijf, Staatsmijnen in Limburg, Geleen (The Netherlands)

(Received July 26th, 1963)

In 1953 JANÁK[1] presented a gas chromatographic analytical method, which differed considerably from the previously used techniques. The new method did not follow the existing practice of measuring the concentrations of the separated components in the carrier gas by means of a suitable detector, but measured the volume of the separated, pure components. The carrier gas was pure carbon dioxide, which, after being passed through the column, was absorbed in a nitrometer filled with potassium hydroxide solution. The volume of the non-absorbed, chromatographically separated sample was measured and recorded as a function of time.

Considering its great accuracy and the ease with which it can be employed, it is surprising that the method has not become more widely adopted. The unpopularity of the method may be put down partly to the drawbacks attached to it. For example, the method is very cumbersome. During analysis the gas volume in the nitrometer must be read at given intervals. The separation of the components is often difficult to observe; one moment's inattentiveness may invalidate the determination. Moreover there are some systematic errors, which can be ruled out only by applying empirical correlation factors; these include the "wall effect", which is caused by KOH solution adhering to the wall of the nitrometer, and the effect of the vapour tension of the solution. As will be made clear in the present paper, all these drawbacks can be overcome in a fairly simple way.

The assets of the method—great accuracy at higher percentages, and the fact that calibration can be dispensed with—make it very attractive, and several investigators have tried to eliminate the drawbacks without sacrificing the advantages.

JANÁK[2] designed an apparatus in which the gas bubbles ascending through the KOH solution collect under a bell suspended on a balance. The displacement of the balance arm is recorded photographically.

VAN DER CRAATS[3] started from an entirely different principle. He washed the gas issuing from the column with a countercurrent of KOH solution, and measured the pressure rise in a partly evacuated collecting vessel.

LEIBNITZ[4] used a nitrometer. The level of the KOH-solution in the nitrometer is kept constant by means of a piston which, via a relay system, is actuated by a contact near the meniscus of the solution. The piston travel is recorded mechanically.

JANÁK[5] constructed a variant of LEIBNITZ's device. He maintained a constant pressure in his fully closed system by withdrawing the KOH solution by means of a piston system operated by a mercury manometer.

VÉLUT[6] described a device in which the nitrometer pressure was kept constant by displacing the KOH-gravity feed vessel. The displacement was recorded.

In addition, a patent application[7,8] was filed for a device in which the carbon dioxide is absorbed in conformity with VAN DER CRAATS's principle, but in which the pressure of the gas is kept constant by means of a piston system.

None of these devices, however, fully satisfied our demands; what we needed was a reliable, accurate and fully automatic instrument that could be operated by an unskilled laboratory technician. On the basis of LEIBNITZ's principle we designed and constructed a device which is suitable for routine analysis of permanent gases and satisfies all our requirements.

PRINCIPLE

The sample, which should not be appreciably soluble in KOH solution, is passed through the column with a stream of carrier gas (very pure carbon dioxide) and collected in a nitrometer fitted with a short stem and containing concentrated KOH solution (see Fig. 1). The carrier gas dissolves in the solution; the sample gas ascends and collects over the liquid, producing a pressure rise, which causes the liquid level to fall. (The system burette–nitrometer–overflow is in fact a sensitive pressure indicator.)

The falling meniscus breaks a contact, which actuates a motor-operated piston-burette. The burette enlarges the volume over the solution by a volume equal to that of the amount of gas fed in, *e.g.* until the contact is just closed again, so that the original pressure is restored.

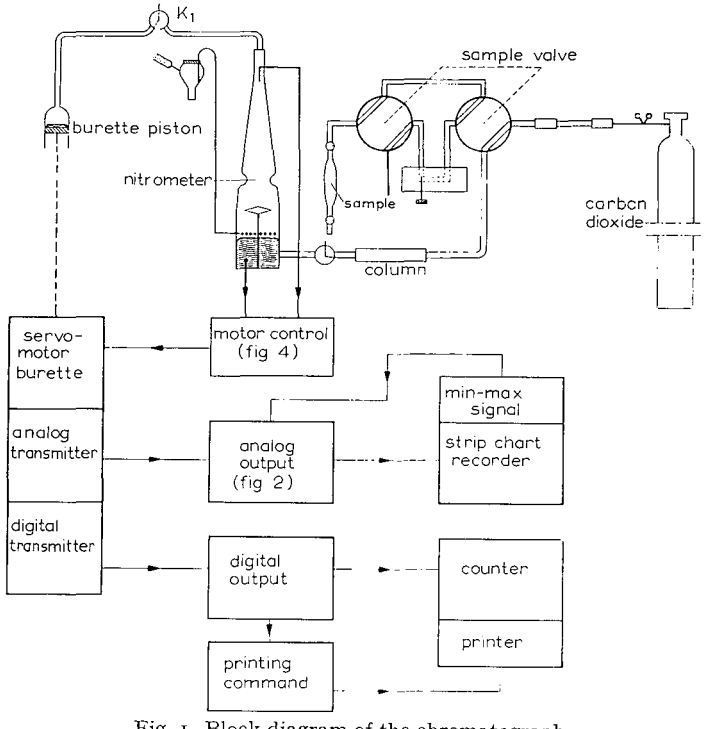


Fig. 1. Block diagram of the chromatograph.

The position of the motor-operated burette, which, as made clear above, is a measure of the amount of component that has issued from the column, can be recorded both on an analog recorder (Fig. 2) and on a digital printer. The whole device is thermostated.

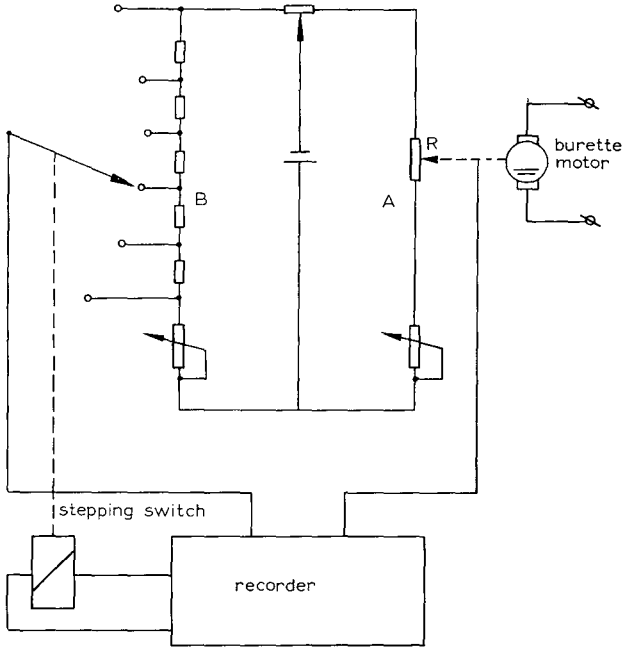


Fig. 2. Analog output circuit.

Discussion

We have found that the instrument satisfies all our requirements. Systematic errors have been eliminated and, as the KOH solution remains at a constant level, the wall effect has been ruled out. At the thermostatically controlled constant temperature the vapor pressure of the KOH is a constant, for which allowance is made in calibrating the instrument. Differences in solubility between the permanent gases in the KOH solution are very small if the concentration of the solution is 46 % and the temperature is adjusted to 35 °C. The instrument is fully automated and operation is therefore simple. After the sample introduction, no further operations are required, so that, as far as ease of handling is concerned, the device is comparable to, say, a katharometer chromatograph. The result of the analysis (in percent) can be read directly from an analog recorder and from a digital printer.

PROCEDURE

The sample is introduced into a sample-feeding system fitted with a six- or eight-way valve and left there for a few moments to adopt the thermostat temperature. The sample compartment is connected with the atmosphere via a capillary and the sample is thus measured at atmospheric pressure.

The volume of the components after leaving the column is also measured at atmospheric pressure and at the same temperature, which eliminates correction for barometer reading and temperature. The volume of the sample compartment is *ca.* 3 ml and can be varied over a limited range (see below).

The sample is fed to the column with a stream of carrier gas consisting of very pure carbon dioxide (> 99.998 % CO_2). The carbon dioxide is purified in the device described by KEULEMANS[9]. In principle, the column can be packed with any suitable material (except molecular sieves). The carrier gas that has left the column is fed to the nitrometer, where the carbon dioxide dissolves and the insoluble components ascend. However, all gases are more or less soluble in KOH; for permanent gases the differences in solubility have been found to decrease with increasing concentration of the KOH solution. The velocity with which the gas bubbles ascend depends on the viscosity of the liquid. The concentration up to which the viscosity remains reasonable is *ca.* 46 %. To avoid deposition of potassium carbonate at these high concentrations, it is essential to keep the temperature of the KOH over 30 °C and to introduce the gas under mercury (Fig. 3). To prevent the gas bubbles from adhering to the mercury surface, the surface is covered with a thin layer of mercuric oxide. In addition, a continuous stream of KOH solution is fed to the nitrometer from storage flask A, via a restriction R. After use, the KOH solution is discharged via overflow B and replaced by fresh solution, which, to avoid foaming, must not contain any Ca or Ba. If traces of these metals should still be present, foaming can be suppressed by adding a small amount of EDTA.

The motor-operated burette is a commercial piston burette (Metrohm E 274) equipped with a Honeywell balancing motor. The motor is operated by means of a platinum contact in the nitrometer stem. When the falling meniscus of the KOH solution in the nitrometer breaks the contact, the motor is started. When the level of the KOH solution rises again upon displacement of the piston, the contact closes and the motor stops. In the course of one analysis this starting–stopping sequence is repeated a great many times. During continuous operation the piston moves at the rate of *ca.* 0.07 ml/sec, which is fast enough for the increase in volume to be reflected in a "peak". The smallest displacement of the piston needed for accurate recording of the "tail" and the blank is 0.003 ml. This is achieved by rapidly braking the motor after the contact has been closed. The principle of this method of motor control is outlined in Fig. 4.

Coil 1 of the two-phase motor is continuously energized with 110 V AC; coil 2 is fed via thyratrons V 1 and V 2. As long as contact C is open (*i.e.* the level of the KOH solution is under the contact point) the current passes via V1 and V 2, because their control grids connect with the cathode via R 1 and R 2. The current passing through R 4 energizes the diode circuit D 3, and thus renders thyratron V 3 nonconducting. When the KOH solution reaches contact C, in other words, when C is closed, the thyratrons V 1 and V 2 are opened via the two diode circuits D 1 and D 2. As the current has now stopped flowing through R 4, V 3 will become conducting (g 3 via R 3 to k 3) and a direct current is passed through motor coil 2. This arrangement permits the motor to be stopped within 40 msec.

The amount of energy needed for operating the circuit is very small, so that undesirable gas evolution at electrode C is avoided.

To restore the burette to its original position, the rotation of the motor can be reversed by means of switch S. This switch is coupled to valve K_1, which serves to vent

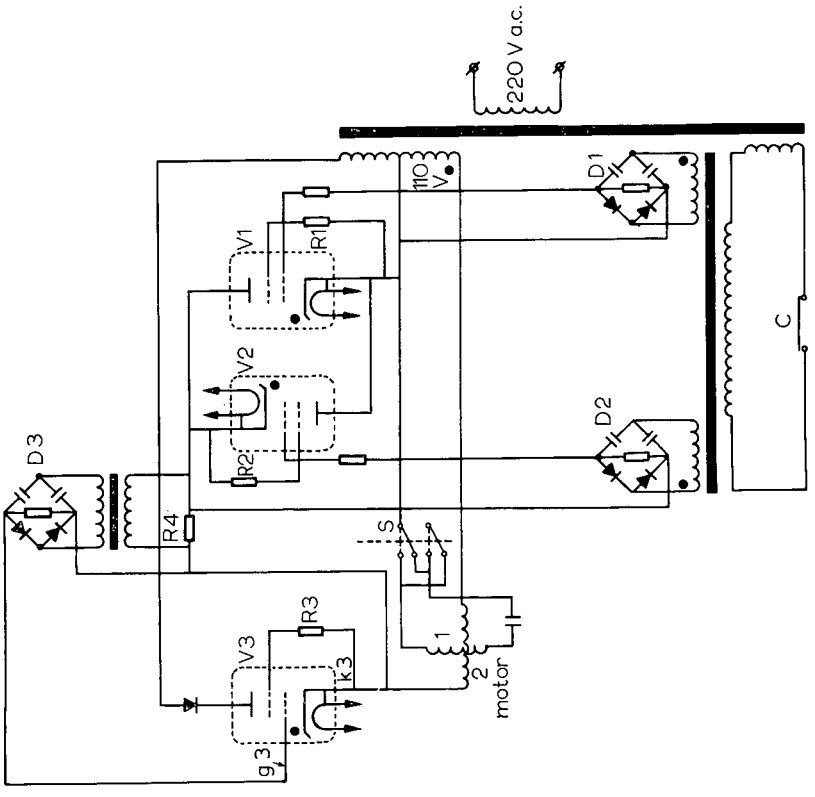


Fig. 4. Motor control circuit.

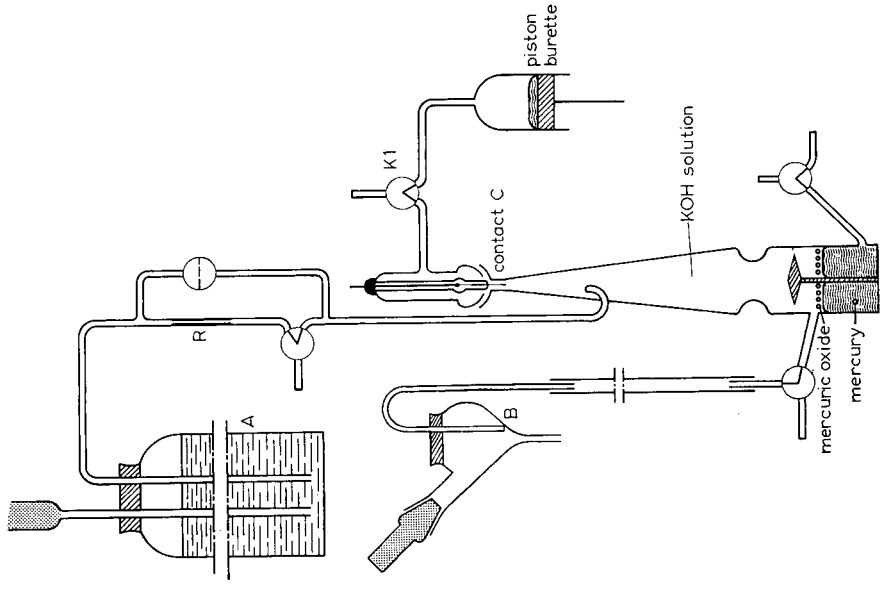


Fig. 3. Nitrometer.

the gas that has collected in the burette. Microswitches in the burette keep the burette within the measuring range.

Besides the burette drive pulley, the motor shaft also drives two gear wheels. One of these is directly coupled to a ten-coil potentiometer which delivers the analogous output signal. The principle of the analogous output can be seen in Fig. 2. Potentiometer R which, as shown above, is coupled to the piston, carries a constant weak current (branch A). Consequently, the voltage taken from the potentiometer is a measure of the volume of the gases that have collected in the nitrometer. This voltage is fed to an mV recorder, so that an integrated chromatogram is recorded on the recorder chart. To improve the reading accuracy and to facilitate reading, the relation between the voltage taken from the potentiometer and the recorder input voltage is so adjusted that 0.1 % of the total sample volume corresponds to 1 mm on the recorder. As the diagram width of the conventional recorders is no more than 250 mm, a second branch B has been incorporated in the circuit. Via this branch a compensating voltage equal to full-scale deflection of the recorder can be applied when the recorder pen has reached the edge of the diagram. This is done automatically by means of a stepping-switch controlled by the recorder pen. The number of opposing millivolts can be read from a digital indicator tube.

The digital measuring circuit is arranged on the other motor-driven gear. This gear carries a disc provided with 100 slots. A lamp over the disc intermittently illuminates a photoresistance under the disc when the latter is being rotated. A counter is energized via a amplifier circuit. The transmission ratio between burette and counter disc is such that 0.1 % of the total sample volume corresponds to 1 counting impulse (one illumination of the photo-resistance).

After elution of a peak, the pulses collected on the counter are printed on a strip chart, after which the counter is zeroed. The printing device becomes operative in response to a signal from a printing command circuit; this signal is given when no counting signals have been received over an adjustable time interval. The printed digits directly give the composition of the sample in 0.1 %.

As the ratio of the transmission from the burette to the digital measuring circuit can only be adjusted discontinuously (the adjustment being determined by the number of teeth on the gearwheels) provision has been made also for a fine adjustment. To this end the sample volume has been made adjustable. By means of a screw that can be fitted in the sampling space of a conventional six- or eight-way sampling valve, the sample volume can be varied over a limited range. The absolute amount of sample need not be known, because the (unknown) sample volume is always taken equal to 100 %, so to 1000 mm in the analog output and to 1000 counts in the digital output.

RESULTS

To determine the accuracy of the method, a number of analyses were performed on a mixture of permanent gases under the following conditions:

Column: inner diameter 8 mm, length 5.5 m.

Column packing: silica gel Ee (Gebr. Herrmann, Cologne, Ehrenfeld, Germany) washed with 3 *N* HCl and activated with a stream of carbon dioxide for 4 h at 250°C. Fraction 0.35–0.42 mm.

TABLE I

ANALYSES OF PURE GASES

	Analog				*Digital*			
	H_2 *mm*	O_2 *mm*	N_2 *mm*	*Ar mm*	H_2 *pulses*	O_2 *pulses*	N_2 *pulses*	*Ar pulses*
Mean	1002.0	1005.5	1005.1	1001.5	601.1	603.6	603.6	601.3
φ	5	5	5	5	5	5	5	5
s	1.5	1.2	0.8	1.8	1.5	1.2	1.4	1.2
$s_{tot.}$		2.0 = 0.20 vol. % φ = 20				1.7 = 0.28 vol. % φ = 20		

s = standard deviation.
φ = degrees of freedom.

Gas flow rate: *ca.* 50 ml/min.
Temperature: *ca.* 35 °C.

At the time of the analyses we had at our disposal only a burette giving 600 pulses for the total volume.

(1) A series of pure gas samples was analyzed. The total deflection of the recorder pen and the printed total number of pulses were considered to correspond to 100 % concentration (see Table I). The figures listed in Table I show that the accuracy of the analysis of a gas mixture is *ca.* 0.20 % by volume (standard deviation $s_{tot.}$). It is only when a greater accuracy is desired that the differences in solubility between the various gases become important.

(2) We then prepared 12 gas mixtures of known compositions. Each mixture contained 2 or 3 components in different ratios, and was analyzed in duplicate by means

TABLE II

RELATIVE AND ABSOLUTE DEVIATION (ANALOG METHOD)

		90 %	*60 %*	*40 %*	*30 %*	*10 %*	*Mean*
H_2	s	0.17	0.20	0.05	0.11	0.14	0.16
	φ	3	7	3	3	3	19
Δ_{mean}		−0.05	−0.12	+0.02	+0.20	+0.10	+0.01
CH_4	s		0.08		0.27	0.14	0.17
	φ		3		3	7	13
Δ_{mean}			0.00		+0.10	+0.02	+0.05
CO	s		0.13		0.17	0.28	0.20
	φ		3		3	3	3
Δ_{mean}			−0.32		−0.02	−0.07	−0.14
Ar	s		0.13		0.32	0.15	0.22
	φ		3		3	3	9
Δ_{mean}			+0.27		−0.02	+0.12	+0.12
Mean	s	0.17	0.16	0.05	0.23	0.18	0.18
	φ	3	16	3	12	16	50
Δ_{mean}		−0.05	−0.06	+0.02	+0.06	+0.04	+0.01

Δ = absolute systematic deviation (vol. %).
φ = degrees of freedom.
s = standard deviation (vol. %).

of the chromatograph. The sample volumes were equal to those in the first test series. The standard deviation and the deviation from the calculated percentage are represented in Tables II and III. These tables show that the absolute systematic deviation (Δ) is insignificant; this also holds for the differences between the accidental errors (s) for the various gases.

TABLE III

RELATIVE AND ABSOLUTE DEVIATION (DIGITAL METHOD)

		90%	60%	40%	30%	10%	Mean
H_2	s	0.17	0.26	0.15	0.23	0.20	0.22
	φ	3	7	3	3	3	19
Δ_{mean}		−0.15	+0.02	+0.17	+0.30	+0.02	+0.07
CH_4	s		0.13		0.21	0.28	0.23
	φ		3		3	7	13
Δ_{mean}			−0.17		−0.12	−0.02	−0.10
CO	s		0.15		0.13	0.35	0.23
	φ		3		3	3	9
Δ_{mean}			−0.37		−0.12	−0.05	−0.18
Ar	s		0.13		0.25	0.10	0.17
	φ		3		3	3	9
Δ_{mean}			+0.33		−0.07	−0.15	+0.03
Mean	s	0.17	0.20	0.15	0.21	0.26	0.22
	φ	3	16	3	12	16	50
Δ_{mean}		−0.15	−0.03	+0.17	0.00	−0.04	−0.03

(3) Finally, one sample of coke oven gas was analyzed a few times on the chromatograph. The results of this analysis are compiled in Table IV.

Table V is derived by combining the figures in Tables I, II, III and IV. The results

TABLE IV

ANALYSES OF ONE SAMPLE OF COKE OVEN GAS

	Analog (%)					Digital (%)				
	H_2	N_2+O_2+Ar	CO	CH_4	Total	H_2	N_2+O_2+Ar	CO	CH_4	Total
1	61.7	4.2	4.5	25.9	96.3	61.8	4.2	4.5	25.8	96.3
2	61.8	4.2	4.6	25.7	96.3	62.0	4.2	4.5	25.8	96.5
3	61.6	4.1	4.7	25.8	96.2	61.8	4.2	4.5	26.0	96.5
4	62.0	4.3	4.6	25.8	96.7	62.0	4.2	4.7	25.8	96.7
5	62.1	4.1	4.6	25.5	96.3	62.0	4.2	4.7	25.6	96.5
6	62.1	4.2	4.7	25.4	96.4	62.0	4.2	4.4	25.5	96.2
7	61.8	4.1	4.7	25.8	96.4	61.8	4.3	4.7	25.8	96.6
8	62.3	4.3	4.7	25.8	97.1	62.1	4.3	4.8	25.8	97.0
9	62.5	4.3	4.7	25.8	97.3	62.3	4.3	4.7	26.0	97.3
Mean	61.9	4.20	4.64	25.72	96.55	61.98	4.23	4.62	25.79	96.62
s	0.29	0.09	0.07	0.17		0.17	0.05	0.12	0.16	
φ	8	8	8	8		8	8	8	8	
s_{mean}		0.18						0.13		
φ_{mean}		32						32		

TABLE V

ACCURACY

	Analog method	*Digital method*
s	0.185 vol. %	0.210 vol. %
φ	102	102

of the analog and digital methods do not differ significantly. It may be taken that if use is made of the burette giving 1000 counts per analysis, the accuracy of the digital method will be slightly greater, because in this case the influence of the rounding-off errors will be relatively smaller.

ACKNOWLEDGEMENTS

The authors express their sincere gratitude to Mr. N. G. L. M. JANSSEN and Mr. P. J. STORNEBRINK of the Central Instrumentation Service, who designed and built the electronic part of the chromatograph. Without their valuable assistance, it would have been impossible to realize the device presented in this paper. Thanks are also due to Dr. T. C. VAN HOEK for his stimulating interest and valuable suggestions.

SUMMARY

This paper describes a gas chromatograph developed on the basis of JANÁK's principle, which permits rapid and accurate determination of the permanent gases in a gas mixture of arbitrary composition. The analytical result is fed both to an analog recorder and to a digital printer. At 102 degrees of freedom, the standard deviation is 0.185 vol. % for the analogous and 0.210 vol. % for the digital measurement. The systematic error lies within the statistical error.

REFERENCES

1 J. JANÁK, *Chem. Listy*, 47 (1953) 1190.
2 J. JANÁK, *Microchim. Acta*, 1956, 1038.
3 F. VAN DER CRAATS, *Anal. Chim. Acta*, 14 (1956) 136.
4 E. LEIBNITZ, H. HRAPIA AND H. G. KÖNNECKE, *Brennstoff-Chem.*, 38 (1957) 14.
5 J. JANÁK AND K. TESAŘIK, *Z. Anal. Chem.*, 164 (1958) 62.
6 M. VÉLUT AND J. JOURDA, *Rev. Inst. Franç. Pétrole Ann. Combust. Liquides*, 13 (1958) 1635.
7 E. LEHRER, *Control Engineering*, 1962, p. 95–97.
8 BADISCHE ANILIN UND SODA FABRIK, *Ger. Pat.*, 1 065 639, 17 Sept., 1959.
9 A. I. M. KEULEMANS, *Gas Chromatography*, edited by C. G. VERVER, Reinhold, New York, 1959, p. 227.

INSECT SEX ATTRACTANTS

IV. THE DETERMINATION OF GYPLURE IN ITS MIXTURES BY ADSORPTION AND GAS CHROMATOGRAPHY*

WILLIAM A. JONES AND MARTIN JACOBSON

Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. (U.S.A.)

(Received August 5th, 1963)

INTRODUCTION

Following the reported synthesis of gyplure [(+)-12-acetoxy-*cis*-9-octadecen-1-ol][2,3], a sex attractant for the male gypsy moth (*Porthetria dispar* (L.)), many samples of this material prepared commercially and in our laboratories were bioassayed under laboratory and field conditions. The results were inconsistent. Some gyplure samples, particularly those prepared commercially, demonstrated only weak attraction and others were completely devoid of activity. Previous findings[4] showed that gyplure could be rendered unattractive by admixture with at least 20% of its crude *trans* isomer; thus, it appeared likely that loss of activity was probably due to the presence of unattractive contaminants formed by failure to rigidly control production conditions. Before we could proceed with confidence, we needed a satisfactory method to determine the gyplure content of such mixtures and, if possible, to identify the major contaminants. These needs were met by the simple adsorption and gas-chromatographic methods described in this report.

MATERIALS**

Solvents

The isooctane and ethyl acetate used were obtained by distilling the technical-grade solvents.

Adsorbents and packings

Merck reagent-grade silicic acid was used for the adsorption chromatography. Commercial SE-30, Carbowax 20M, and Chromosorb W (acid-washed, 60–80 mesh) were used for gas chromatography.

Gyplure

Gyplure for this study was prepared in our laboratories according to the procedure previously reported,[3] except that the ricinoleyl alcohol (b.p. 145–8°/0.15 mm, n_D^{25} 1.4711) used as starting material was obtained by careful fractional distillation of a

* For Report III in this series, see ref. 1.

** The mention of trade names or products does not constitute endorsement by the Department of Agriculture over those not named.

commercial product (Archer-Daniels-Midland Adol 40) by use of a spinning-band column. Commercial batches of gyplure had been prepared by acetylating distilled Adol 40, selectively saponifying the undistilled diacetate, distilling the final product and decolorizing it by filtration through charcoal.

Standard reference substances

Ricinoleyl alcohol was obtained as described above. Oleyl and stearyl alcohols were commercial materials obtained from Archer-Daniels-Midland Co. and Eastman Kodak Co. (White Label, No. 4053, m.p. 56.5–8.0°), respectively. Oleyl acetate was prepared by acetylation of the commercial alcohol with acetyl chloride in dry benzene, and was not distilled. Pure 1,12-diacetoxy-*cis*-9-octadecene (b.p. 167–8°/0.1 mm, n_D^{26} 1.4515) was prepared in 90% yield.[3]

Gas-chromatographically pure gyplure was prepared by selectively saponifying the diacetate[3] and chromatographing the distilled product (b.p. 163–4°/0.1 mm, n_D^{26} 1.4595; 89% yield) on a column of silicic acid (see below); elution with 10% ethyl acetate in isooctane following elution with 5% ethyl acetate in isooctane gave a 76% yield of the pure product.

Analysis. Calculated for $C_{20}H_{38}O_3$: C, 73.56; H, 11.74. Found: C, 74.57; H, 11.97.

Gas-chromatographically pure 1-acetoxy-*cis*-9-octadecen-12-ol was prepared by the following procedure. A solution containing 10 g ricinoleyl alcohol (b.p. 145–8°/0.15 mm), 2 g glacial acetic acid, 0.3 ml sulfuric acid, and 100 ml dry benzene was refluxed for 2 h with a trap to continuously distill the water as it was formed. Ether was added to the cooled solution and the separated organic layer was washed successively with 5% hydrochloric acid, 5% potassium hydroxide, and water, then dried over sodium sulfate. Removal of the solvent left 10 g yellow oil, 1.132 g of which was chromatographed on a column of 80 g silicic acid. The column was eluted with 400 ml of isooctane containing 2% ethyl acetate and then with 400 ml of isooctane containing 5% ethyl acetate; removal of solvent from the latter eluate gave 0.661 g of the desired product (n_D^{25} 1.4607).

Analysis. Calculated for $C_{20}H_{38}O_3$: C, 73.56; H, 11.74. Found: C, 72.94; H, 11.23.

METHODS

Column chromatography

The column used for isolating gyplure from its mixtures was prepared by the following procedure. A slurry of 80 g silicic acid was poured into a glass tube (38 × 2.5 cm) fitted with a stopcock and glass wool plug at the lower end and a 24/40 female joint at the top. Air pressure of 3 p.s.i. was applied to the column to pack the adsorbent, which was then prewashed successively with 150 ml of 70% ethyl acetate in isooctane and 200 ml of isooctane.

Commercial gyplure (1 g) dissolved in a few milliliters of isooctane was placed on the column and washed in with a few portions of isooctane. The column was eluted successively with 400 ml of isooctane (fraction 1), 400 ml of 5% ethyl acetate in isooctane (fraction 2), 400 ml of 10% ethyl acetate in isooctane (fraction 3), and 400 ml of 25% ethyl acetate in isooctane (fraction 4). Eluates were collected in 100-ml portions. Table I gives the percentage content of each fraction found in four replicates of the same commercial sample; the average contents of these fractions were 8.6, 34.3, 30.4, and 21.2%, respectively.

TABLE I

PERCENTAGE OF FRACTIONS 1–4 OBTAINED BY COLUMN CHROMATOGRAPHY OF COMMERCIAL GYPLURE

Sample No.	*Fraction 1*	*Fraction 2*	*Fraction 3*	*Fraction 4*
1	9.1	32.8	31.2	20.4
2	6.8	31.5	32.7	22.0
3	8.4	33.5	29.8	21.7
4	10.0	39.5	28.0	20.5
		Average		
	8.6	34.3	30.4	21.2

Fraction 1 (8.6% of the original commercial sample) was shown, by gas chromatography described below, to contain oleyl acetate (peak 3) and two unidentified minor components (peaks 4 and 7), and was not investigated further.

Fraction 2 (34.3%) was shown by gas chromatography to contain oleyl alcohol (peak 1), stearyl alcohol (peak 2), 1-acetoxy-*cis*-9-octadecen-12-ol (peak 9), 1,12-diacetoxy-*cis*-9-octadecene (peak 10), and an unidentified component (peak 5). Fraction 2 dissolved in a few milliliters of 1% ethyl acetate in isooctane was placed on an 80-g silicic acid column (prepared and prewashed as above) and washed in with a few portions of the same solvent. The column was then eluted successively with 400 ml of this solvent (fraction 2A), 400 ml of 2% ethyl acetate in isooctane (fraction 2B, collected in sixteen 25-ml portions), 400 ml of 3% ethyl acetate in isooctane (fraction 2C), and 400 ml of 5% ethyl acetate in isooctane (fraction 2D, collected in 25-ml portions). Fraction 2A, comprising 8% of fraction 2, contained 21% oleyl acetate (peak 3) and 60% unidentified material (peak 7). Fraction 2B, comprising 56% of fraction 2, contained 16% oleyl alcohol (peak 1), 5% stearyl alcohol (peak 2), 10% unidentified material (peak 5), and 66% 1,12-diacetoxy-*cis*-9-octadecene (peak 10); its elution pattern is shown in Fig. 1. The eighth 25-ml portion of fraction 2B, after removal of solvent at 20 mm pressure, consisted of 95% pure 1,12-diacetoxy-*cis*-9-octadecene (peak 10).

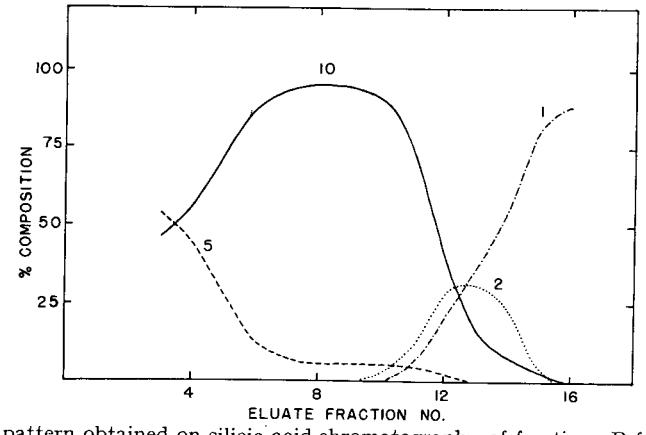


Fig. 1. Elution pattern obtained on silicic acid chromatography of fraction 2B from commercial gyplure. Numbers correspond with the peak numbers given for the compounds in Table II.

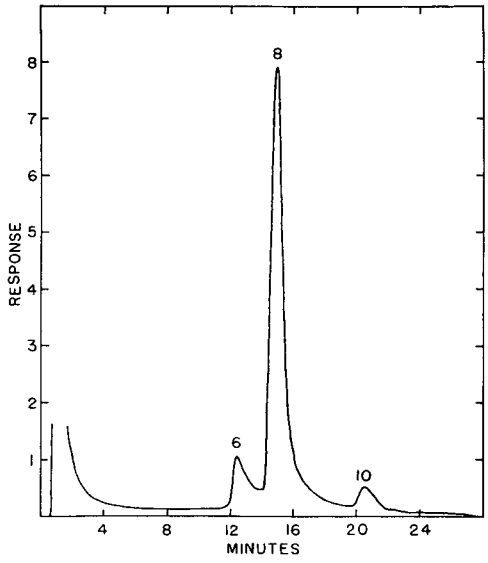


Fig. 2. Gas chromatogram of laboratory-synthesized gyplure. Sample size, 1 μl of a 3% solution in acetone; temperature, 200°; nitrogen flow rate, 22 ml/min; stationary phase, SE-30 on a support of acid-washed Chromosorb W (60–80 mesh), 5% by weight; stainless steel column 8ft. by 1/8 in. O.D. Peak numbers correspond with those given for the compounds in Table II.

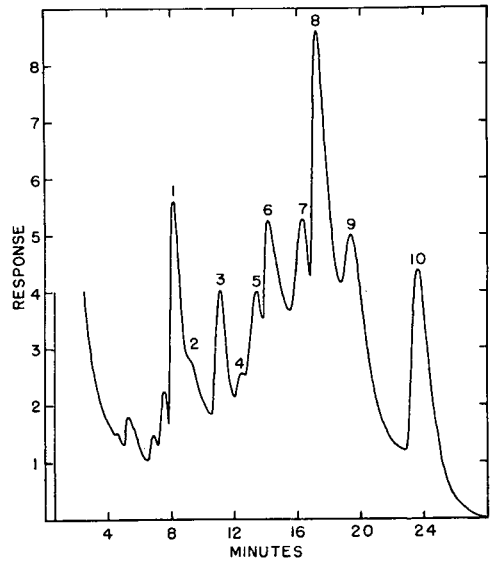


Fig. 3. Gas chromatogram of commercial gyplure. Conditions as given for Fig. 2, except that the nitrogen flow rate was 20 ml/min. Peak numbers correspond with those given for the compounds in Table II.

Analysis. Calculated for $C_{22}H_{40}O_4$: C, 71.69; H, 10.94. Found: C, 71.43; H, 10.71.

Fraction 2C, comprising 19% of fraction 2, contained 84% oleyl alcohol (peak 1). Fraction 2D, comprising 17% of fraction 2, consisted of pure 1-acetoxy-*cis*-9-octadecen-12-ol (peak 9) after removal of solvent.

Analysis. Calculated for $C_{20}H_{38}O_3$: C, 73.56; H, 11.74. Found: C, 74.13; H, 12.12.

Fraction 3 (30.4%) consisted of pure 12-acetoxy-*cis*-9-octadecen-1-ol (gyplure) (peak 8 by gas chromatography).

Analysis. Calculated for $C_{20}H_{38}O_3$: C, 73.56; H, 11.74. Found: C, 73.85; H, 11.90.

Fraction 4 (21.2%) consisted of pure ricinoleyl alcohol (peak 6 by gas chromatography).

Analysis. Calculated for $C_{18}H_{36}O_2$: C, 75.99; H, 12.76. Found: C, 75.72; H, 12.30.

Gas chromatography

All chromatograms were obtained with an F & M Model 1609 gas chromatograph equipped with a flame ionization detector, with nitrogen as the carrier gas. Columns used were 8-ft., 1/8-in. diameter stainless steel packed with 5% SE-30 on 60–80 mesh, acid-washed Chromosorb W, and 10-ft., 1/4-in. diameter aluminum packed with 5% Carbowax 20M on 60–80 mesh, acid-washed Chromosorb W.

Gyplure samples prepared in these laboratories by the procedure described above contained predominantly gyplure (peak 8) with small amounts of ricinoleyl alcohol (peak 6) and 1,12-diacetoxy-*cis*-9-octadecene (peak 10), as shown in Fig. 2. In contrast with this, commercial samples tested were found to contain varying amounts of the 10 substances obtained by column chromatography, as shown in Fig. 3. Retention times of these substances with their peak numbers and their relative retention ratios based on gyplure for both type columns are given in Table II.

TABLE II

RETENTION TIMES AND AVERAGE RELATIVE RETENTION RATIOS (GYPLURE = 1.00) OF SUBSTANCES OBTAINED BY GAS CHROMATOGRAPHY OF COMMERCIAL GYPLURE

Peak No.	*Compound*	*Retention times (min) on*		*Average relative retention ratios on*	
		SE-30	*Carbowax 20M*	*SE-30*	*Carbowax 20M*
1	Oleyl alcohol	7.4	10.7	0.49	0.31
2	Stearyl alcohol	7.9	9.8	0.54	0.28
3	Oleyl acetate	9.8	9.8	0.66	0.28
4	Unidentified	10.5	14.4	0.73	0.41
5	Unidentified	11.8	21.8	0.80	0.63
6	Ricinoleyl alcohol	12.3	41.4	0.83	1.19
7	Unidentified	14.0	18.0	0.96	0.52
8	12-Acetoxy-*cis*-9-octadecen-1-ol	14.7	34.8	1.00	1.00
9	1-Acetoxy-*cis*-9-octadecen-12-ol	16.7	36.0	1.13	1.03
10	1,12-Diacetoxy-*cis*-9-octadecene	20.2	30.0	1.37	0.86

Conditions: For SE-30, columns were 8 ft. stainless steel, 1/8 in. O.D., 5% on acid-washed Chromosorb W (60–80 mesh), N_2 flow rate 22 ml/min., temperature 200°. For Carbowax 20M, columns were 10 ft. stainless steel, 1/4 in. O.D., 5% on acid-washed Chromosorb W (60–80 mesh), N_2 flow rate 27 ml/min., temperature 210°.

DISCUSSION

The chromatographic methods described permit the ready determination of actual gyplure content in laboratory and commercial pilot-plant samples of gyplure, as well as the separation of the pure *cis* isomer from its contaminants. Samples prepared in the laboratory showed an approximate content of 80% or more *cis*-gyplure, contaminated with unchanged ricinoleyl alcohol and its diacetate. On the other hand, commercial samples contained approximately 30–35% *cis*-gyplure, the contaminants being stearyl, oleyl, and ricinoleyl alcohols, oleyl acetate, ricinoleyl alcohol diester, 1-acetoxy-*cis*-9-octadecen-12-ol, and three unidentified substances. It is apparent that, in order to obtain a satisfactory grade of gyplure, it is necessary to use a pure grade of ricinoleyl alcohol and to distill the product obtained at each stage.

Although satisfactory resolution of the samples by gas chromatography is obtained with both types of column packing, SE-30 permits much shorter retention times than Carbowax 20M.

SUMMARY

Adsorption and gas-chromatographic methods are described for determining the content of *cis*-gyplure in samples of gyplure prepared in the laboratory and the pilot plant, as well as for separating the *cis* isomer from its contaminants.

REFERENCES

[1] M. JACOBSON, *J. Org. Chem.*, 27 (1962) 2670.
[2] M. JACOBSON, *J. Org. Chem.*, 25 (1960) 2074.
[3] M. JACOBSON AND W. A. JONES, *J. Org. Chem.*, 27 (1962) 2523.
[4] M. JACOBSON, *Advan. Chem. Ser.*, No. 41 (1963) 1.

A RAPID METHOD FOR THE ESTIMATION OF TOTAL 11-DEOXY-17-OXOSTEROIDS IN URINE

B. S. THOMAS AND R. D. BULBROOK

Division of Chemistry and Biochemistry, Imperial Cancer Research Fund, London (Great Britain)

(Received July 17th, 1963)

The main components of the 11-deoxy-17-oxosteroid fraction of human urine are 3β-hydroxy-androst-5-ene-17-one (dehydroepiandrosterone, DHA), 3α-hydroxy-5α-androstan-17-one (androsterone) and 3α-hydroxy-5β-androstan-17-one (aetiocholanolone). Separate estimation of these compounds by paper chromatography or gradient elution from alumina is time-consuming and for certain purposes measurement as a group may serve equally well. This paper describes a rapid method for such an estimation based on modifications of the methods of SAVARD[1] and KELLIE AND WADE[2].

MATERIALS AND APPARATUS

Chemicals

All solvents were of analytical reagent grade, except for "heptane" ("fraction from petroleum" B.D.H.) and were redistilled shortly before use. Ethanol (Burroughs Ltd., A. R. Grade) was kept in the dark over *m*-phenylenediamine for 7 days and then redistilled twice. Neutral alumina (Woelm) was deactivated with 6% water (w/v). β-Glucuronidase (Powder B) was prepared by the method of DODGSON AND SPENCER[3] from limpets (*Patella vulgata*) and assayed by the method of DODGSON, LEWIS AND SPENCER[4]. The activity found was approx. 900,000 units/g. *m*-Dinitrobenzene (B.D.H. "purified for 17-KS determinations") was further purified by the method of CALLOW, CALLOW AND EMMENS[5]. Ethanolic KOH (2.5 *N*) was prepared by the method of WILSON AND CARTER[6] and stored under N_2 at −20°. Sodium acetate–acetic acid buffer solution (2.5 *M*, pH 4.0) was kept as stock solution and diluted to 0.5 *M* before use.

Chromatographic apparatus

The chromatographic apparatus is shown in Figs. 1 and 2. It is constructed of aluminium alloy and consists of a circular platform (A) supported on three legs (B), two of which have adjustable feet for levelling purposes. Around the top of the platform and 0.2 cm in from the outer edge is channelled a gully (C) 0.5 cm wide and 0.2 cm in depth. This acts as a reservoir and ensures an even supply of eluant to the chromatography papers. Circumscribing the legs and 1½ in. above the base is fitted a circular ring (D) for supporting 10 tapered graduated 10 ml test tubes. On top of the platform and of similar diameter is a detachable ring (E), in the centre of which fits a 4 in. diameter Petri dish (F). The ring is fixed to the platform by means of knurled screws (G).

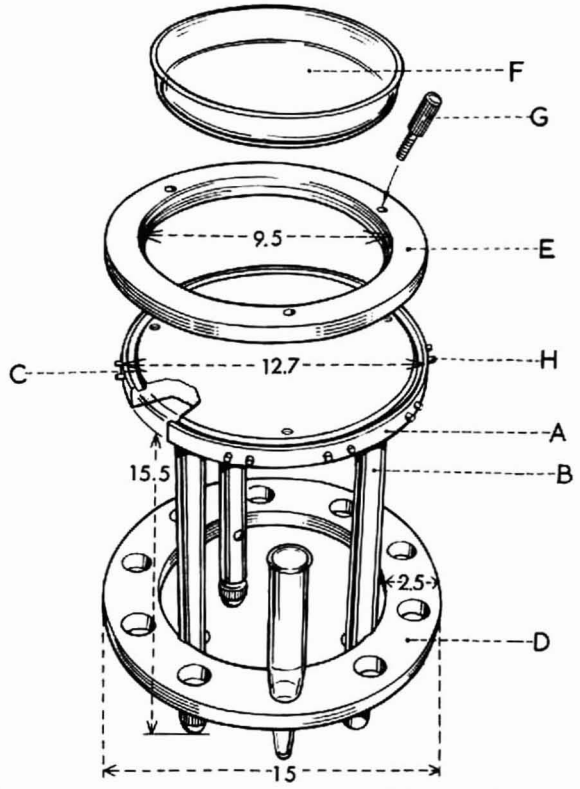


Fig. 1. Exploded diagram of chromatographic apparatus. The important measurements (in cm) are given in the figure or in the text. All other dimensions are not critical. The alphabetical key is explained in the text.

A circular disc of Whatman glass fibre paper is cut to 12 cm diameter and is fitted between the detachable ring (E) and the platform (A), and beneath the Petri dish. Two strips of glass fibre paper (2.5 cm wide) run under the Petri dish and cross at right-angles at the centre. The ends of these strips are looped over the edges of the Petri dish so that they clear the sides (a circular metal supporting frame protruding 2–3 mm above the rim of the dish is helpful but not essential). The ends of the strips are weighed down and held in place by a square of glass in the centre of the dish (see Fig. 2). If the wet paper strips which feed solvent to the circular disc of glass fibre paper touch the edge of the dish when it is filled with developing solvent there is excessive capillary flow.

Chromatography

Strips of Whatman No. 4 paper are shaped as shown in Fig. 3. The strips are dipped into a freshly prepared solution of 30 % (v/v) propylene glycol in methanol. They are removed with forceps, shaken to remove excess liquid and hung up by a corner for 10 min at room temperature to allow the methanol to evaporate. Before application of the steroid extracts, the papers are sandwiched between four strips of glass plate leaving exposed an area 0.5 cm either side of the start line.

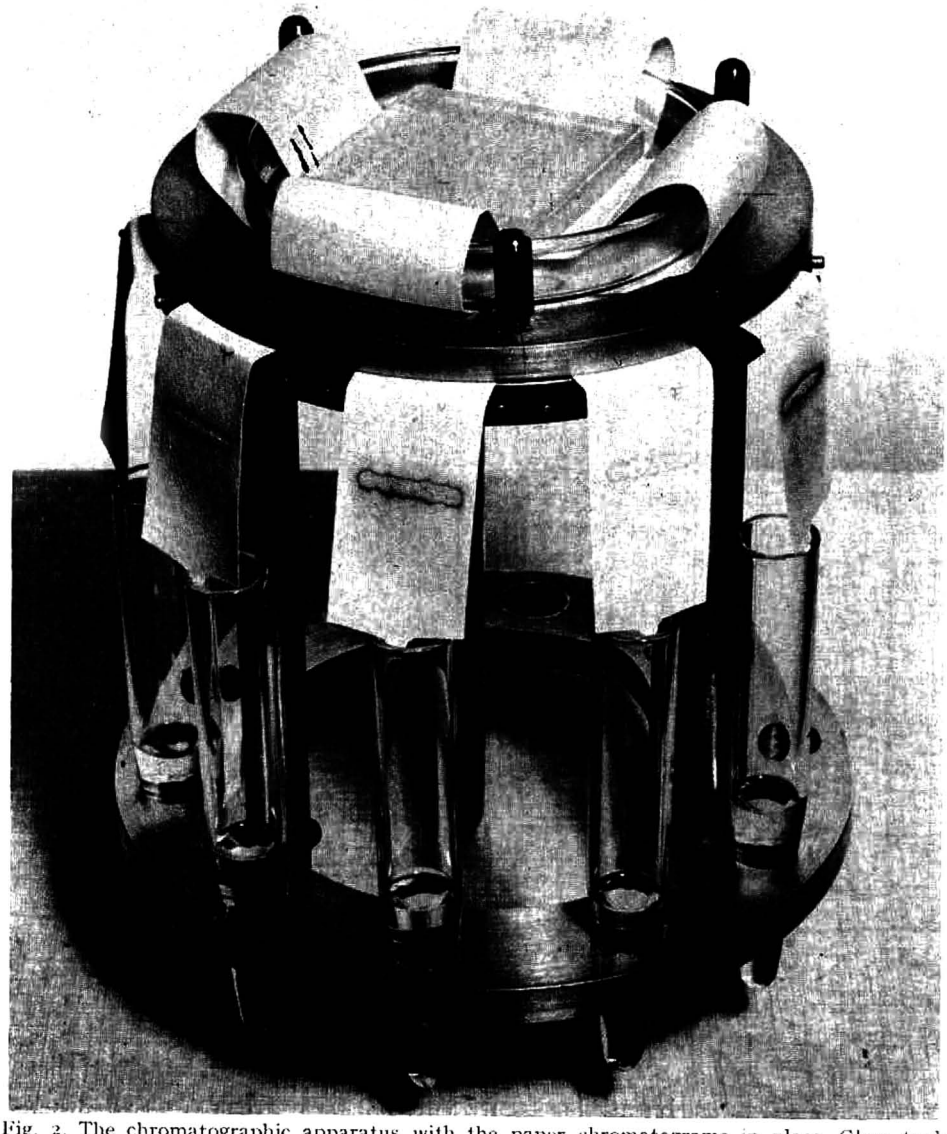


Fig. 2. The chromatographic apparatus with the paper chromatograms in place. Glass tank not shown.

Urine extracts (see subsequent section for preparation) in tapered tubes are dissolved in approx. 50 μl of benzene–ethanol (1:1) mixture and applied along the start line using a pipette with a capillary end. At least 2 mm clear space must be left at each end of the start line or the steroids will streak down the edges of the chromatogram during the run. Three further applications each of approx. 20 μl of solvent are necessary to transfer the extract quantitatively to the paper strip. Drying between each application is carried out with a stream of cold air.

The strips are then left for 10 min to allow a backflow of propylene glycol displaced by the application of the benzene–ethanol mixture. This backflow narrows the band of extract at the start line.

The strips are then bent at right angles approximately half-way between the top and the start line. The top flap so formed is inserted with forceps between the detachable ring (E) and the platform of the elution rack (A), making sure that each

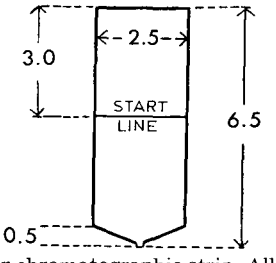


Fig. 3. Diagram of paper chromatographic strip. All measurements are in cm.

paper is spaced between the protruding pins (H) on the side of the platform. The chromatograms must protrude about 2 mm from the side of the platform or capillary action will cause excessive flow of solvent. The small tongue at the end of the chromatogram (Fig. 3) should rest just inside the rim of the collection tube. The knurled screws are tightened and the whole rack is placed in a circular glass jar (approx. 8 in. high, 8 in. diameter), the atmosphere of which has been equilibrated with heptane by placing about 50 ml of this solvent in the tank at least 30 min previously.

Heptane (35–40 ml) is then poured into the Petri dish, a lid placed on the jar, and the chromatogram is over-run until 3.5–4.0 ml of eluate has collected in the graduated test tube. Initial equilibration of the chromatogram is unnecessary. At a temperature of 20–25° the solvent drips from the chromatogram at a rate of 1–1.2 ml/h. It may occasionally be necessary to recharge the Petri dish with heptane during the run.

THE METHOD IN DETAIL

(1) *Extraction of conjugated 17-oxosteroids*

24 h urine specimens are made up to 2 l with water if below this volume and two 50 ml aliquots taken. To each is added ammonium sulphate (25 g) which is stirred until dissolved. The urine is then extracted with ether–ethanol (3:1) mixture (3 × 25 ml). The extracts are pooled and filtered through a fluted 15 cm Whatman No. 4 paper into a 250 ml round-bottomed flask. Using a rotary evaporator the extract is taken to near dryness under partial vacuum at the water pump at a temperature less than 45°. Ethanol (5 ml) is added and the full vacuum applied. The dry extract is dissolved in ethanol (4 ml) and transferred to a tapered centrifuge tube graduated at 10 ml. Three further washes of ethanol (2, 2 and 1 ml) are transferred from the flask to the centrifuge tube which is then made up to the 10 ml mark with ethanol. After mixing, the tube is centrifuged for 3 min at 1400 g and an 8 ml aliquot of supernatant (equivalent to 40 ml original urine) is pipetted into a 50 ml tube and blown to dryness under N_2 at 45° (*cf.* EDWARDS, KELLIE AND WADE[7]).

(2) Enzymic hydrolysis and solvolysis: estimation of total 17-oxosteroids

β-Glucuronidase powder (approx. 25 mg, depending on the activity) is homogenized with acetate buffer (10 ml, 0.5 *M*, pH 4.0) and then centrifuged at 1400 *g* for 5 min. The supernatant (which contains the bulk of the enzyme activity) is decanted into a measuring cylinder and made up with buffer solution to give a final concentration of approx. 2000 units/ml. This solution (5 ml) is added to the dry conjugate extract and stood overnight at 40°. NaCl (4 g) is then added, followed by water (15 ml). The solution is then taken to pH 1 (glass electrode) with 4 *N* H_2SO_4 (approx. 0. 25 ml) and then extracted with ethyl acetate (2 × 20 ml). These extracts are pooled and incubated at 50° for 4 h (*cf.* BURSTEIN AND LIEBERMAN[8]).

The volume of the ethyl acetate, which now contains the steroids freed by β-glucuronidase hydrolysis and by solvolysis, is reduced to approx. 5 ml under N_2 at 45°. Benzene (35 ml) is added, the mixture transferred to a separating funnel and washed (3 × 5 ml) with a freshly prepared solution of sodium dithionite (5 % w/v, in *N* NaOH) until any red pigment present (indirubin) is decolourized. The organic phase is then washed with water (2 × 2.5 ml) and taken to dryness (rotary evaporator). The extract is transferred with ethanol (1 × 2 ml, 3 × 1 ml) to a test tube, taken to dryness under N_2 and redissolved in 1.0 ml ethanol. A single aliquot (0.2 ml) is removed and the total 17-oxosteroids estimated by the method of CALLOW *et al.*[5] except that after 1 h at 25°, the reaction mixture is diluted with only 5 ml of 95 % ethanol. The colours are read at 440, 520 and 600 mμ and a correction applied (ALLEN[9]).

(3) Separation and estimation of the 11-deoxy-17-oxosteroid fraction

From the results of the estimation of the total 17-oxosteroids, the content in the remaining 0.8 ml of ethanol solution can be calculated. From this a further aliquot containing 30–60 μg of 17-oxosteroid is taken into a small tapered tube, blown to dryness with N_2 and transferred to the paper chromatogram and run as previously described. The eluate from the chromatogram which contains the 11-deoxy-17-oxosteroid fraction is taken to dryness under N_2 at 50° and the Zimmerman reaction carried out as previously described.

(4) Separation and estimation of the 11-oxygenated 17-oxosteroid fraction

The method can also be used for the estimation of the 11-oxygenated 17-oxosteroids by subtracting the results of the determination of the 11-deoxy fraction from those obtained for the total 17-oxosteroids. Alternatively, the paper chromatograms may be transferred to a second chromatographic tank in which the mobile phase is toluene–methylene chloride (1:1). The 11-oxygenated fraction is eluted from the paper when 3.0 ml of eluant have been collected in the test tube. This method does not remove stationary phase from the paper. The Zimmerman reaction is then carried out as for the 11-deoxy fraction except that half quantities of reagents are used and the final reaction mixture is diluted with 2.5 ml of 95 % ethanol.

In a subsequent section, independent evidence is given for the validity of results obtained with the method for the 11-deoxy fraction. Since similar evidence is at present not as complete for the 11-oxygenated fraction, the method for this group of compounds will not be discussed further.

(5) *General comments on the method*

(*a*) *Extraction of conjugates and hydrolysis.* The method of EDWARDS *et al.*[7] must be used if enzymic hydrolysis is to be complete in 18 h. Furthermore, the extraction of the conjugates results in a substantial saving in the amount of enzyme used. Centrifugation of the solution of enzyme in acetate buffer removes a considerable portion of inactive protein and reduces the chances of subsequent formation of emulsions. The free steroids released by hydrolysis with limpet enzyme are extracted together with unhydrolysed sulphates into ethyl acetate and the latter are subsequently cleaved by solvolysis (BURSTEIN AND LIEBERMAN[8]).

Interference by urinary pigments is reduced considerably by shaking the extract obtained after hydrolysis with alkaline dithionite, and the bulk of any remaining pigments remain on the start line of the chromatogram. Occasionally a red pigment is found which is not reducible with dithionite. This runs with the solvent front and can be eluted with the first two drops of heptane and discarded without loss of 17-oxosteroids. Evaporation of most of the ethyl acetate extract and addition of benzene results in a more efficient removal of pigments by alkaline dithionite.

(*b*) *Chromatography.* The behaviour of various components of the 17-oxosteroid fraction during paper chromatography and their elution from the strips by overrunning is shown in Fig. 4.

These results show that there is a considerable safety margin between the complete elution of DHA (the most polar of the 11-deoxy-17-oxosteroid fraction) and the start of the elution of 11-keto-androsterone, the least polar of the 11-oxygenated

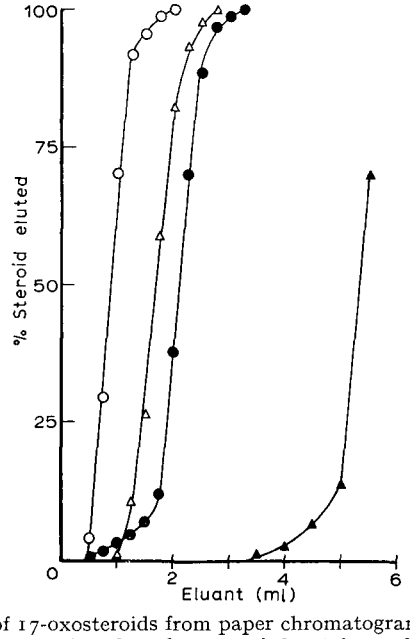


Fig. 4. The elution curves of 17-oxosteroids from paper chromatograms. Pure standards were run on paper chromatograms (see text) and analyses carried out for each steroid for each 0.25 ml of eluant. For 11-keto-androsterone the analyses were done for each 0.5 ml of eluant. —O—O—, androsterone; —△—△—, aetiocholanolone; —●—●—, DHA; —▲—▲—, 11-keto-androsterone.

17-oxosteroids normally encountered in urine. In some urine specimens from pregnant women androst-4-ene-3,11,17-trione is present and this compound runs between DHA and 11-keto-androsterone, partly eluting into the 11-deoxy fraction.

RESULTS

(1) *Evaluation of the method*

The definition of the terms used in this section and the details of the calculations have been described by BROWN, BULBROOK AND GREENWOOD[10]. The accuracy of the method, measured by recovery experiments, is given in Table I. These experiments show that pure steroids added at various stages of the method, either free or unconjugated, can be recovered in amounts of 75 % or over of the original starting material.

TABLE I

RECOVERY OF PURE STEROIDS ADDED AT VARIOUS STAGES IN THE METHOD

Steroid added	*Amount (μg) of steroid added to:*			*No. of estimations*	*Mean recovery (%)**	*Range (%)*
	Paper	*Hydrolysed urine extract*	*Urine*			
DHA Androsterone Aetiocholanolone	25–40	—	—	20	98.2 (±2.72)	93–103
DHA Androsterone Aetiocholanolone	—	10	—	18	95.3 (±6.89)	84–112
DHA Androsterone Aetiocholanolone	—	—	40	12	85.7 (±7.46)	75–99
$Na \cdot DHA \cdot SO_4 \cdot 2H_2O$**	—	—	50	11	78.8 (±16.25)	62–107
Paper blanks	—	—	—	5	0.005***	—
11-OH-Aetiocholanolone	30	—	—	4	97.3	91–102
11-OH-Aetiocholanolone	—	30	—	4	84.8	81–99
11-OH-Aetiocholanolone	—	—	40	4	73.0	71–74

* Figures in brackets represent standard deviations.
** Obtained from Steraloids Ltd.
*** Corrected optical density.

The precision of the method was calculated from differences between duplicate estimations in a series of 51 assays on different urine specimens. At a mean level of 6.68 mg of 11-deoxy-17-oxosteroids/24 h the estimate of the standard deviation(s) is 0.43 mg/24 h. From this it can be calculated that the lower limit of sensitivity of the method is about 0.8 mg/24 h (if duplicate determinations are performed).

(2) *Comparison of results with those obtained by gradient elution from alumina*

In 36 instances, aliquots of urine were taken after the hydrolytic procedures already described had been carried out. The amounts of DHA androsterone, and aetiocholanolone in one aliquot were then determined by the method of KELLIE AND WADE[2] and the amounts of total 11-deoxy-17-oxosteroid in a second aliquot were determined

by the paper chromatographic method described. The results of the comparison are shown in Fig. 5. In a further 36 instances, the comparisons were made by processing aliquots of the original urine completely separately by either method (also shown in Fig. 5). These comparisons show clearly that the titre of total 11-deoxy-17-oxosteroids

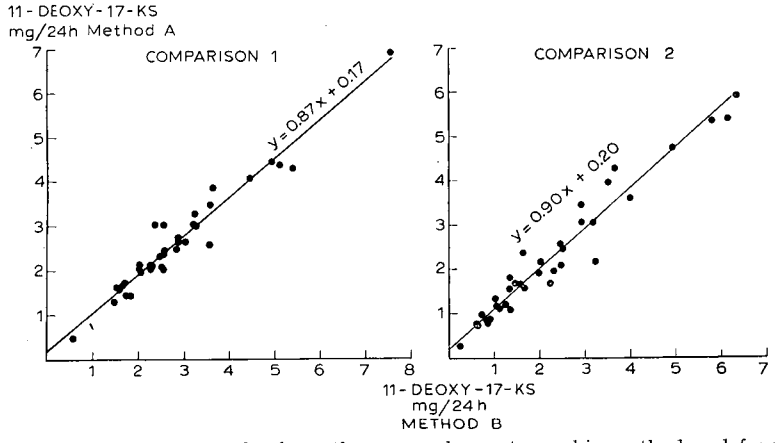


Fig. 5. Comparison between results from the paper chromatographic method and from gradient elution from alumina. Method A refers to the paper chromatographic method. The results of determinations of DHA, androsterone and aetiocholanolone by gradient elution (method B), expressed in terms of DHA standard were summed for this comparison. Comparison 1: hydrolytic procedure common to both methods. Comparison 2: separate processing throughout (see text). The lines are the fitted regression curves of the paper chromatography result (y) on the gradient elution results (x).

obtained by the paper chromatographic method can be almost entirely accounted for by summing the amounts of DHA, androsterone and aetiocholanolone determined by gradient elution from alumina. The correlation coefficients (r) are 0.978 and 0.980 respectively.

DISCUSSION

Various methods have been described for the separation and determination of individual 17-oxosteroids in urine (SAVARD[1], KELLIE AND WADE[2], BUSH AND WILLOUGHBY[11], BROOKS[12]). All are time-consuming and require a high degree of skill for constant reliability. In some clinical investigations the amounts of the individual 11-deoxy-17-oxosteroids may not be required and all that is needed is a measurement of the total 11-deoxy fraction. The paper chromatographic method described here is suitable for this purpose. It is especially suitable for handling large numbers of determinations and a trained worker can conveniently assay 12 urine specimens in duplicate in one week. It is also useful as a preliminary purification step for subsequent separation of the 17-oxosteroids by paper chromatography and may be useful in this context for gas chromatography.

The use of 6.5 cm strips obviates the need for paper blank corrections which may be considerable if 3 MM paper of conventional size is used. Recoveries of 17-oxosteroids from the short strips have been consistently higher in our hands than from 3 MM paper. Also use of the latter sometimes results in incomplete resolution of the

less polar from the more polar 17-oxosteroids (see BUSH AND WILLOUGHBY[11], BUSH[13], JAMES[14]).

ACKNOWLEDGEMENTS

We are very much indebted to Mr. T. H. NEWTON of Netherne Hospital, Coulsdon, for his help in designing and making the chromatography apparatus. We would like to thank Miss ANN COLWIN for her skilled technical assistance, Miss R. A. A. SALOKANGAS for advice and help and the Medical Research Council Steroid Reference Collection for the generous gift of some of the compounds used in this work.

SUMMARY

(1) A paper chromatographic method is described for the rapid separation and estimation of the 11-deoxy-17-oxosteroids in urine. The method may also be useful for the measurement of the 11-oxygenated compounds.

(2) The method is based on extraction of conjugates from urine, enzymic hydrolysis and solvolysis followed by paper chromatography in a specially designed apparatus on 6.5 cm strips of paper impregnated with propylene glycol.

(3) The results are comparable with those obtained by summing the individual components of the 11-deoxy-17-oxosteroid fraction separated and estimated by gradient elution from alumina.

REFERENCES

1 K. SAVARD, *J. Biol. Chem.*, 202 (1953) 457.
2 A. E. KELLIE AND A. P. WADE, *Biochem. J.*, 66 (1957) 196.
3 K. S. DODGSON AND B. SPENCER, *Biochem. J.*, 55 (1953) 315.
4 K. S. DODGSON, J. I. M. LEWIS AND B. SPENCER., *Biochem. J.*, 55 (1963) 253.
5 N. H. CALLOW, R. K. CALLOW AND C. W. EMMENS, *Biochem. J.*, 32 (1938) 1312.
6 H. WILSON AND P. CARTER, *Endocrinology*, 41 (1947) 417.
7 R. W. H. EDWARDS, A. E. KELLIE AND A. P. WADE, *Mem. Soc. Endocrinol.*, No. 2 (1953) 53.
8 S. BURSTEIN AND S. LIEBERMAN, *J. Biol. Chem.*, 233 (1958) 331.
9 W. M. ALLEN, *J. Clin. Endocrinol.*, 10 (1950) 71.
10 J. B. BROWN, R. D. BULBROOK AND F. C. GREENWOOD, *J. Endocrinol.*, 16 (1957) 41.
11 I. E. BUSH AND M. WILLOUGHBY, *Biochem. J.*, 67 (1957) 689.
12 R. V. BROOKS, *Biochem. J.*, 68 (1958) 50.
13 I. E. BUSH, *The Chromatography of Steroids*, Pergamon Press, London, 1961, p. 339.
14 V. H. T. JAMES, *J. Endocrinol.*, 22 (1961) 195.

A METHOD FOR THE DETECTION AND ISOLATION OF TRACES OF ORGANIC FLUORINE COMPOUNDS IN PLANTS

ROBERT H. WADE, JAMES M. ROSS AND HARRIS M. BENEDICT

Physical Sciences Laboratories, Southern California Laboratories, Stanford Research Institute, South Pasadena, Calif. (U.S.A.)

(Received July 16th, 1963)

INTRODUCTION

During the course of several years of investigating atmospheric fluoride pollution problems we have felt the need for two separate fluoride analytical method. First, a rapid semiquantitative method for the determination of excess amounts of fluoride in vegetation has been needed; such a method, recently developed in these laboratories, is to be reported elsewhere[1]. Second, a method for the detection and isolation of microgram quantities of organic fluorine compounds from plant materials is the subject of the present paper. The method is presented in two parts: a rapid procedure for screening large numbers of plant extracts and materials for the presence of any organic fluorine compounds, and a chromatographic process for further isolation and identification of materials found in the screening method.

Background

Fluoride analyses of vegetation are confined for the most part to modifications of the WILLARD AND WINTER[2–4] method. Although this method is reliable and reproducible, it is time consuming, requires relatively large samples (1–5 g vegetation containing at least 5 μg of fluoride) and, unless special precautions are taken, is not likely to account for fluorine present in the organic form. At best it gives a total fluoride (organic and inorganic value. The isolation and identification of fluorine compounds in plant materials to be used as salad crops or as feed for grazing animals is important because of the known fluoride uptake from polluted atmospheres and because of the very wide range of toxicities, especially of organic fluorine compounds. These toxicities range all the way from inert, innocuous materials (such as the Freons) to poisons (such as fluoroacetic acid and some of the fluoro-olefins where the LD_{50} is in the range that makes detection difficult or impossible). For example, inhalation of the refrigerant Freon CF_2Cl_2 (Freon 12) of 20 % concentration does not even produce unconsciousness, although analgesia and confusion result. Recovery is complete within ten minutes[5]. In contrast, the lethal dose of fluoroacetic acid for man is estimated at 2–10 mg per kg[6,7] body weight. The toxicity of inorganic fluoride is intermediate between these extremes; the toxic dose of sodium fluoride is estimated between 5 and 10 g, an amount unlikely to be ingested from vegetable contamination. Regular intake of sublethal doses of inorganic fluoride, however, may produce a definite and damaging series of chronic symptoms[8–11]. It was the concern over these chronic symptoms and

the possibility of the presence of even more toxic organic fluorine compounds in fluoridated salad and forage crops that initiated this research.

In attacking these problems, forage plants (alfalfa and orchard grass) and leafy vegetables (chard, spinach and romaine lettuce) were fumigated with concentrations of about 0.60–0.83 $\mu g/m^3$ of hydrogen fluoride during their entire growth period. These concentrations produced no fluoride-type markings on the leaves, although a relatively high concentration of fluorides accumulated in them (30–100 p.p.m.).

The problems of separating organic from inorganic fluorides were first attacked qualitatively by making several series of extractions, using solvents of widely varying polarity. Regardless of whether the solvents were employed in order of increasing or decreasing polarity, most (over 90 %) of the fluoride was found in the most polar solvent (water), indicating the fluoride to be essentially inorganic. However, sufficient fluoride was found in the non-polar solvents to allow at least the possibility of the presence of organic fluorine compounds.

DISCUSSION

The isolation and identification of organic fluorine compounds from plant or animal materials is complicated by two factors. First, not only is the fluorine present in very small amounts, but it has previously been shown to be present largely in the inorganic form. As a consequence, organic fluorine in fluoride-fumigated plants is present in even smaller amounts, if at all, and hidden by the small but nevertheless swamping amount of inorganic fluoride. Second, organic fluorine compounds do not in themselves constitute a functional class; for example, fluoroacids behave essentially like other acids and fluoroalcohols have the properties of alcohols in general. Consequently, the chemistry of organic fluorine compounds cannot be used in any way to isolate them as a group nor as tests to indicate their presence. Therefore, every fraction, extract, or compound must be completely freed of inorganic fluoride and then quantitatively analyzed for residual fluorine.

Quantitative analytical methods are all based on the determination of fluoride in ionic form. A large variety of methods for conversion of organic fluoride to analyzable ionic form habe been reported. Many conventional methods are summarized by SIMONS[12]. None of them are applicable in the microgram range and all are too tedious and time consuming for large numbers of samples. In 1955, SCHÖNIGER[13] introduced an oxygen flask combustion method for reducing many of the elements in organic compounds to ionic or other analyzable forms. From this rapid and inexpensive method, a very suitable procedure for the determination of fluorine in organic compounds has been developed. It is particularly applicable to the quantitative determination of fluorine in spots developed on paper chromatograms. However, in a search for minute amounts of unknown organic fluorine compounds in the presence of much larger amounts of inorganic fluoride, new procedures were needed.

METHOD

The various solvent and aqueous extracts of fluoridated vegetation were concentrated on a rotovacuum apparatus and first analyzed for total fluoride.

Sub-micro determination of fluorine

A modification of the procedure reported by SOEP[14] was used for quantitative sub-micro determination of fluorine. We found, as did SOEP, the decoloration of the zirconium-Eriochrome Cyanine R complex to be proportional to fluoride concentration. We also investigated the more sensitive aluminum-morin complex[15] and a number of other metal-dye complex reagents such as sodium 2-(*p*-sulfophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonate-zirconium lake (SPADNS)[16] before standardizing on the Eriochrome complex as most satisfactory for the determination of from 0–3 μg of fluoride. A new direct color alizarin complexan method of fluoride analyses reported by BELCHER *et al.*[17–19] was not found satisfactory for this work but was used in the semiquantitative procedure to be reported elsewhere[1].

Apparatus

A 125-ml iodine flask modified with a platinum wire attached to the stopper according to SCHÖNIGER[13] plus a microstopcock attached to the bottom of the flask (Fig. 1).

Volumetric flasks, 50 ml size.

Micropipettes, graduated variety 1–5 λ, 5–25 λ and 20–100 λ sizes.

Portable hair dryer for drying spotted strips of paper.

Beckman model DK Ratio Recording Spectrophotometer used at 546 mμ.

Cuvette from Lumetron Photoelectric Colorimeter.

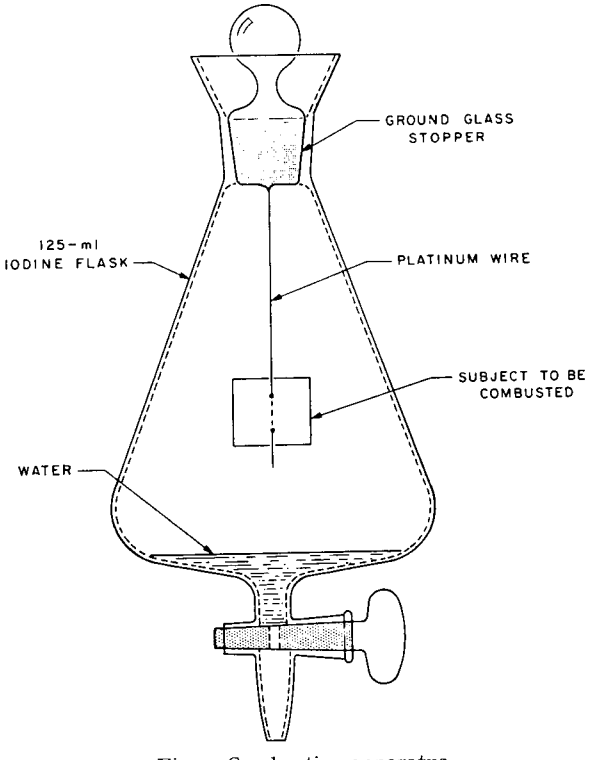


Fig. 1. Combustion apparatus.

The 1-cm cell was small enough to allow adequate filling with 5 ml of solution and was used sideways to give a 32-mm light path. A simple support was provided to hold it in the light path of the Beckman instrument.

Reagents

Solution A: 528.8 mg of Eriochrome Cyanine R dissolved and diluted to 250 ml with water.

Solution B: 76.3 mg of zirconyl chloride octahydrate dissolved in 202 ml of hydrochloric acid (concentrated) and diluted to 500 ml with water.

Reagent: One volume of A mixed with two volumes of B, fresh daily.

Organic fluorine standard: 15.5 mg of sodium difluoroacetate dissolved and diluted to 100 ml with water giving 0.5 μg of fluorine/μl of solution.

Laboratory distilled water was upgraded by distilling through an all-quartz, two-stage water purification apparatus.

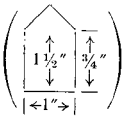
All materials were stored in polyethylene bottles.

PROCEDURE

A portion of each extract (about 100 μl) is spotted on a small piece of filter paper. The paper is dried with hot air from the hair dryer and fixed to the platinum wire of the stopper. After adding 2 ml of water, the flask (Fig. 1) is thoroughly flushed with oxygen and the paper strip is ignited from a small pilot flame and plunged into the flask. We found that when the paper is ignited from the top (in the position to be lowered into the flask) a much larger piece containing more sample can be smoothly combusted. When the flame is at the bottom of the strip as it is plunged into the flask it immediately flashes up consuming the whole of the paper and usually causes some of the paper to fall to the water incompletely burned. Also, the more rapid combustion often causes the stopper to be dislodged with resultant loss of gases. The flask is shaken vigorously, drained, shaken with a second 2 ml of water, and rinsed with about 0.5 ml of water into a 5-ml volumetric flask. The freshly mixed reagent is added (0.40 ml) and the total volume diluted to 5.0 ml. The extinction values are measured at 546 mμ after one hour, using water in the reference beam. The values obtained are compared with a calibration curve prepared similarly from the organic fluorine standard ($CF_2HCOONa$) to obtain the fluorine content. Under these conditions the curve is linear from 0 to about 2.5 of 3 μ of fluoride.

Separation of organic fluoride

Rapid screening procedure. If the above total fluoride analysis (organic or inorganic) shows fluorine to be present, a separation must be accomplished to determine if any of it is in the organic form. The method used for this separation was developed from chromatographic procedures for separation of inorganic anions and from the elution concentration scheme of DAVIS, DUBBS AND ADAMS[20] and was designed to screen large



numbers of plant extracts. First, a small section was cut from a 1-in. roll of Whatman No. 1 chromatographic paper and a small volume of extract (volume dependent on previous total fluoride analysis) spotted along the base of the wedge. The sample can be placed either in a spot or along a line across the base. We did not find it necessary to elute contaminants on the paper and to trim off the tip before using, as did DAVIS *et al.*, because, although materials were found there, they did not interfere with the subsequent fluoride analysis. We found it convenient to use full length microscope slides rather than cut them in half as did DAVIS *et al.* After clamping with No. 2 ring clips, obtainable in any stationery store, ten units can be placed in a standard staining jar without support (Fig. 2). A chromatographic solvent or solvent mixture in which

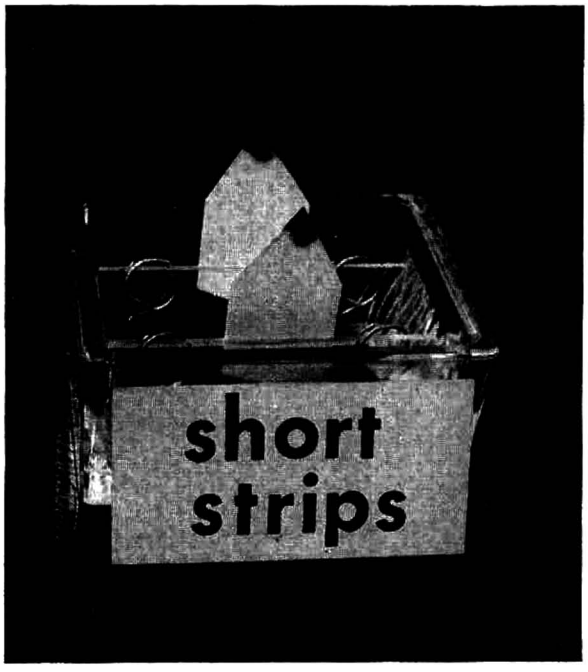


Fig. 2. Short-strip separations of organic from inorganic fluoride.

fluoride ion has been determined not to move ($R_F = 0$) is placed in the jar. The solvent immediately fills the space beteen the two slides, providing a continuous source to the base of the strip. The edges of the dish are covered to reduce evaporation from other than the tips, and the strips allowed to stand overnight. The solvent evaporates from the tip allowing a continual flow of fresh solvent and thereby causing even the slow moving (R_F low) substances to move to the tip and be concentrated there while inorganic fluoride remains on the original spot. Several solvent mixtures for the separation of the halides and other anions are listed by MCOMIE AND POLLARD[21]. We used as standard the upper layer of a mixture of equal parts of *n*-butanol and 1.5 *N* ammonia, well shaken and allowed to stand at least overnight before separation. This solvent mixture was exhaustively tested to be sure that the R_F factor for inorganic fluoride was consistently zero. In addition, papers spotted with sodium fluoride were

run alongside every batch of plant extracts and the fluoride was found in all cases to remain on the original spot. After overnight development, a small portion of the tips and the original spot are separately combusted in the oxygen flask and analyzed for fluoride as above. Under these conditions, inorganic fluoride remains exactly on the original spot and all of the organic fluorine compounds tested move quickly to the tip (Table I). Any fluoride found in the combustion of the tips (with butanol solvent) is therefore definite indication of the presence of a fluoro-organic. As confirming evidence of organic fluorides, duplicate strips were run using water as solvent rather than butanol–ammonia. Here, of course, inorganic fluoride quickly moves to the tip. The movement of fluoro-organics with water is not so straightforward and depends on the nature of the compounds. Highly polar, water-soluble compounds would move with the solvent and non-polar insoluble materials would remain in the original spot. The butanol and water strips together give an indication of the presence and nature of an organic fluorine compound. Table I indicates the results of organic and inorganic fluorides run on short-strip analysis.

TABLE I

SHORT-STRIP FLUORIDE ANALYSIS

Compound or extract	*Solvent*	*Fluoride (μg)*	
		Tip	*Spot*
Perfluorobutyric acid	Butanol	3.2	0.3
Perfluorobutyric acid	Water	3.2	0.2
Sodium fluoroacetate	Butanol	3.0	0
Sodium fluoroacetate	Water	3.5	0.1
Sodium difluoroacetate	Butanol	0.8	0.2
Sodium fluoride	Butanol	0	2.9
Sodium fluoride	Water	3.6	0.1
2,4-Dinitrofluorobenzene	Butanol	0.6	0.3
2,4-Dinitrofluorobenzene	Water	0.9	0

Profile analyses of chromatograms for organic fluorine compounds. It should be obvious, of course, in the short-strip analysis with butanol–ammonia, that essentially all of the organic compounds present move to the tip and not merely the fluoro-organics. Thus, an absolute separation of inorganic from organic fluorine compounds has been obtained, but no indication of the number or nature of the organic compounds present.

If a positive indication of fluoro-organics is obtained in the short-strip analysis, a number of conventional paper chromatographic strips are run. For example, a portion of an extract showing an indication of an organo-fluorine compound in the short-strip analysis is spotted on a 1 in. × 18 in. strip of Whatman No. 1 chromatographic paper. The spot is dried and the strip hung in a chromatographic tank overnight in equilibration with the vapors of the lower butanol–1.5 *N* ammonia layer mentioned above. The strip is then developed several hours with the upper layer of the same solvent mixture and thoroughly dried. Theoretically, the strip could be treated with a suitable reagent to make visible the organic fluoride sought. Unfortunately several factors preclude this: first, as mentioned above, there are no color reactions for the

general fluoro-organic class; second, the compounds, if any, are present in extremely small amounts; third, the paper chromatogram is rather completely stained yellow and green with chlorophyll and other pigments.

Instead, the chromatogram is subjected to a procedure we have chosen to call a profile analysis. It must be quantitatively analyzed for fluorine by sections. A short chromatogram of 12–15 cm is cut crosswise in ten strips, each representing 0.1 R_F factor. A large strip might be cut in twenty strips of R_F 0.05 each. Each strip is combusted in the oxygen flask and analyzed for fluoride. A profile of the fluoride content of the strip is then obtained by plotting the fluoride analyses *versus* the R_F factor (Fig. 3). In the chromatogram represented in Fig. 3A a sample of sodium di-

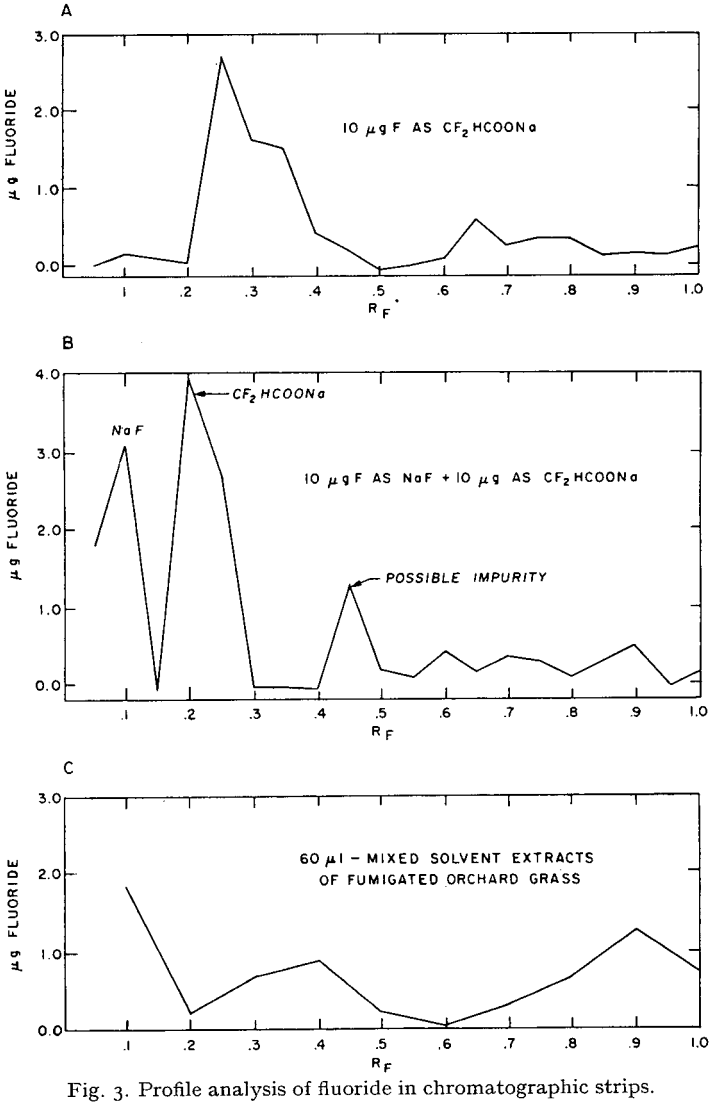


Fig. 3. Profile analysis of fluoride in chromatographic strips.

fluoroacetate containing 10 μg of fluorine was spotted and developed as described. The dried paper was analyzed in twenty strips, each representing 0.05 R_F units. As indicated in the figure, the R_F factor for $CF_2HCOONa$ is about 0.25. The strips at R_F 0.25, 0.30 and 0.35 indicate a total of about 7 mg of fluorine, or 70% recovery. In the chromatograph represented in Fig. 3B, a mixture of sodium fluoride and sodium difluoroacetate were spotted together and developed, again in butanol–ammonia, but a little longer for better separation. The inorganic fluoride is found in the R_F 0.05 and 0.10 strips and the organic fluorine again found at R_F 0.20–0.25. The strip at R_F 0.15 is free of fluorine, indicating complete separation of the two compounds. Fig. 3C shows the profile analysis of a solvent extract of hydrogen fluoride fumigated orchard grass indicating the presence of inorganic fluoride and possible traces of two organic fluorine compounds. Since the strip are cut from the chromatograph by R_F number and not by visual location of any spot, a single compound will usually be found in two or three adjacent strips rather than a single one.

The final step is to isolate the material in pure form. An obvious method is to prepare a number of chromatograms under identical conditions, determine the location of the unknown on one chromatogram by profile analysis and extract the material from similar R_F portions of the remainder. This method was applied as follows. A portion of fumigated orchard grass tips (fluoride previously shown to concentrate there) was extracted in a soxhlet with petroleum ether followed by ether and several other solvents. The ether extract was found to contain 1.2 μg total fluoride per 100 μl. A short-strip analysis indicated the fluoride to be approximately 70% organic. Therefore, a portion of the ether extract was spotted on a 1 in. $\times$ 18 in. strip of paper, equilibrated, and developed with butanol–ammonia. Combustion of the resulting chromatogram in twenty strips (0.05 R_F units) gave a peak at R_F 0.0–0.1 for inorganic fluoride and a larger peak at R_F 0.7–0.9 indicating organic fluoride. To confirm the finding of organic fluoride at R_F 0.7–0.9, eighteen strips $3^1/_4$ in. wide were each spotted with 500 γ of the extract and developed in butanol–ammonia as before. The 0.7–0.9 portion of each strip was cut out, taking care to eliminate most of the chlorophyll portions which run with the solvent front. These portions were extracted with ether in a soxhlet and the extract concentrated to about 2 ml. This extract was found to contain 6–8 μg total fluoride per 100 μl. Short-strip analysis indicated it all to be organic.

Larger quantities of these fluoridated salad and forage crops were investigated by chromatography using heavier preparative papers and cellulose column techniques. Although microgram quantities of organic fluorine compounds were found, the levels were far too low for isolation and identification or to cause concern over toxic materials.

ACKNOWLEDGEMENTS

This work was undertaken as part of a project entitled "The Physical and Chemical Fate of Fluorides in Plants", supported by grants from the National Institutes of Health; Agricultural Research Center, Stanford Research Institute; Columbia Geneva Division, United States Steel Corporation; and Kennecott Copper Corporation. The work was coordinated with the Agricultural Research Center of Stanford Research Institute.

SUMMARY

A method for the detection and isolation of sub-microgram quantities of organic fluorine compounds from plant materials in the presence of much larger amounts of inorganic fluoride is presented. The procedure consists first of a rapid screening step for use with large numbers of vegetable samples and extracts and, second, of a chromatographic step to isolate and characterize any fluoro-organics found. These methods are developed in light of specific chemical characteristics of organic fluorine compounds as a general class. A modification of SOEP's quantitative sub-micro fluoride analytical method is presented as applicable to these isolation methods. Microgram quantities of organic fluorine compounds were found in the plant materials investigated but at a level too low for isolation and identification.

REFERENCES

1 J. M. ROSS, R. H. WADE AND H. M. BENEDICT, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 907.
2 H. H. WILLARD AND O. B. WINTER, *Ind. Eng. Chem., Anal. Ed.*, 5 (1933) 7.
3 L. F. REMMERT, T. D. PARKS, A. M. LAWRENCE AND E. H. MCBURNEY, *Anal. Chem.*, 25 (1953) 450.
4 Stanford Research Institute, Analytical Section, *Fluoride Determination*, ASM 15.06, Menlo Park, Calif., July 1, 1955.
5 M. STACEY, J. C. TATLOW AND A. G. SHARPE, *Advances in Fluorine Chemistry*, Vol. 2, Butterworths, Washington, D.C., 1961, p. 207.
6 M. STACEY, J. C. TATLOW AND A. G. SHARPE, *Advances in Fluorine Chemistry*, Vol. 2, Butterworths, Washington, D.C., 1961, p. 198.
7 F. L. M. PATTISON, *Toxic Aliphatic Fluorine Compounds*, Elsevier, New York, 1959.
8 M. STACEY, J. C. TATLOW AND A. G. SHARPE, *Advances in Fluorine Chemistry*, Vol. 2, Butterworths, Washington, D.C., 1961, p. 186.
9 J. L. SHUPE, *Lab. Invest.*, 8 (1959) 1190.
10 J. L. SHUPE, E. G. FRAME AND M. L. NUNER, *A.M.A. Arch. Ind. Health*, 21 (1960) 348.
11 J. L. SHUPE, E. G. FRAME AND M. L. NUNER, *A.M.A. Arch. Ind. Health*, 21 (1960) 346.
12 J. H. SIMONS, *Fluorine Chemistry*, Vol. II, Academic Press, New York, 1954.
13 W. SCHÖNIGER, *Mikrochim. Acta*, (1955) 123.
14 H. SOEP, *J. Chromatog.*, 6 (1961) 122.
15 J. BOUMAN, *Chem. Weekblad*, 51 (1955) 33.
16 E. BELLACK AND P. J. SCHOUBOE, *Anal. Chem.*, 30 (1958) 2032.
17 R. BELCHER, M. A. LEONARD AND T. S. WEST, *J. Chem. Soc.*, (1958) 2390.
18 R. BELCHER AND T. S. WEST, *Talanta*, 8 (1961) 853.
19 R. BELCHER, M. A. LEONARD AND T. S. WEST, *Talanta*, 2 (1959) 92.
20 F. DAVIS, C. E. DUBBS AND W. S. ADAMS, *Anal. Chem.*, 34 (1962) 175.
21 J. F. W. MCOMIE AND F. H. POLLARD, *Chromatographic Method of Inorganic Analysis*, Academic Press, New York, 1953.

CHROMATOGRAPHIC STUDIES ON AMINOACYL-ACCEPTOR RNA USING PAPER SHEETS OF DIETHYLAMINOETHYL-CELLULOSE

K. BRUCE JACOBSON AND SUSUMU NISHIMURA*

*Biology Division, Oak Ridge National Laboratory***
Oak Ridge, Tenn. (U.S.A.)

(Received September 2nd, 1963)

INTRODUCTION

Partition paper chromatography was used by BENDICH AND ROSENKRANZ[1] to fractionate DNA according to molecular size. Since substituted celluloses are used extensively for column fractionation of nucleic acids, the paper sheet forms of anion-exchange cellulose offer further possibilities for separations of this kind. In unpublished experiments, N. B. FURLONG AND F. J. BOLLUM of this laboratory established that oligodeoxyribonucleotides up to 100 nucleotide units would migrate on DEAE-cellulose paper. Since amino acid-acceptor RNA is of this approximate size, its behavior on various anion-exchange cellulose papers was investigated.

Many investigators have fractionated acceptor RNA from larger molecular weight RNA on ion-exchange cellulose columns; several have observed the separation of one specific type of amino acid-acceptor RNA from the main portion or a change in the ratio of 2 or 3 amino acid-specific acceptor activities[2–4]. Other fractionation technics, such as countercurrent distribution[5,6], chromatography on methylated albumin[7] or hydroxylapatit[8], and column electrophoresis[9] also have been successful in separating amino acid-specific RNA's.

The present work demonstrates that paper chromatography also fractionates acceptor RNA according to amino acid-specific types and describes a number of conditions that affect the chromatographic behavior of RNA. A preliminary note on this subject has been presented[10].

MATERIALS AND METHODS

Preparation of the paper

Two sheets, $8^1/_2 \times 22$ in. of DEAE-cellulose paper (Whatman, DE-20) were clamped on a glass frame for descending chromatography (Kurtz-Miramon frames, Kensington Scientific Co., Berkeley, California) and irrigated successively with 200 ml 1 *M* ammonium bicarbonate, 200 ml water, 200 ml 0.1 *N* HCl and water until no acid could be detected by taste (less than 0.005 *N*). The ammonium bicarbonate seemed to be more satisfactory than NaCl in removing the yellow-colored impurities. The papers were dried, stored at room temperature, and used within three weeks.

* Rockefeller Foundation Fellow.
** Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

Condition for chromatography of [^{14}C]-amino acyl-RNA

Prior to application of the sample, the paper was clamped on a glass frame for descending chromatography and placed in 0.05 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, until it was wet several cm past the origin. About 15 min prior to application of sample, the paper was taken out of the solution to reduce the moisture content at the origin area. The sample was applied and the paper placed in the first developing solution, 0.2 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, which causes mononucleotides to move with an R_F of 0.4–0.6 and most amino acids to move even faster. This served to move out the radioactive amino acid and adenine nucleotides leaving the RNA at the origin. After 2 h the developing solution was changed to 0.58 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, to cause the RNA to migrate; NaCl concentrations less than 0.4 *M* failed to move acceptor RNA. To provide a reference for the rate of the migration of the RNA, a nucleotide was spotted at the origin when development with the higher salt solution was started. A pH of 5.6 was chosen to minimize the spontaneous hydrolysis of the RNA-amino acid ester bond. As will be pointed out later, some chromatograms were developed at room temperature and some at 4°. For fractionation of acceptor RNA's, the lower temperature was used routinely. Radioactive areas were located and measured quantitatively by a 4 π, windowless, gas-flow strip scanner coupled with an integrating unit (Vanguard Instrument Co., LaGrange, Illinois).

Preparation of [^{14}C]-amino acyl-acceptor RNA

The amino acyl-acceptor RNA was prepared from *Escherichia coli* by the method of OFENGAND *et al.*[3], except that DEAE-cellulose column was used in place of ECTEOLA-cellulose. RNA concentration was calculated from the extinction at 260 mμ, assuming that 1 mg RNA/ml in 0.01 *N* NaOH has an extinction coefficient of 30 in a 1 cm light path[3,11]. The enzyme mixture was prepared from the 100,000 $\times$ *g* supernatant of *E. coli* extracts by first adding streptomycin, 1 % final concentration, discarding the precipitate, and fractionating the supernatant with ammonium sulfate. The enzyme fraction precipitated between 0.5 and 0.65 % saturation with ammonium sulfate[12]. The enzyme and RNA were dialyzed and stored at —20°. The reaction mixture (0.5 ml) consisted of 0.1 *M* Tris chloride buffer (pH 7.3 at 25°) or cacodylate buffer pH 7.0, 0.01 *M* Mg acetate, 0.01 *M* ATP, approximately 0.2 μC [^{14}C]-amino acid, 0.25 mg acceptor RNA, and the enzyme preparation. After 15 min at 37°, the reaction mixture was acidified to pH 5 with acetic acid and stored at —20°. For chromatography, aliquots of 0.01–0.35 ml were added as a single spot the wet DEAE-cellulose paper. Since the RNA has a higher affinity for the DEAE than ATP or other components of the reaction mixture, it forms a compact spot not more than 2 cm in diameter with the largest aliquot.

Uniformly labeled [^{14}C]-amino acids were obtained commercially and were of specific activities greater than 50 mC/mmole.

RESULTS

Resolution of RNA into amino acid-specific types

RNA esterified with different radioactive amino acids was chromatographed on DEAE-cellulose paper, and the separation achieved for four amino acyl-RNA's is

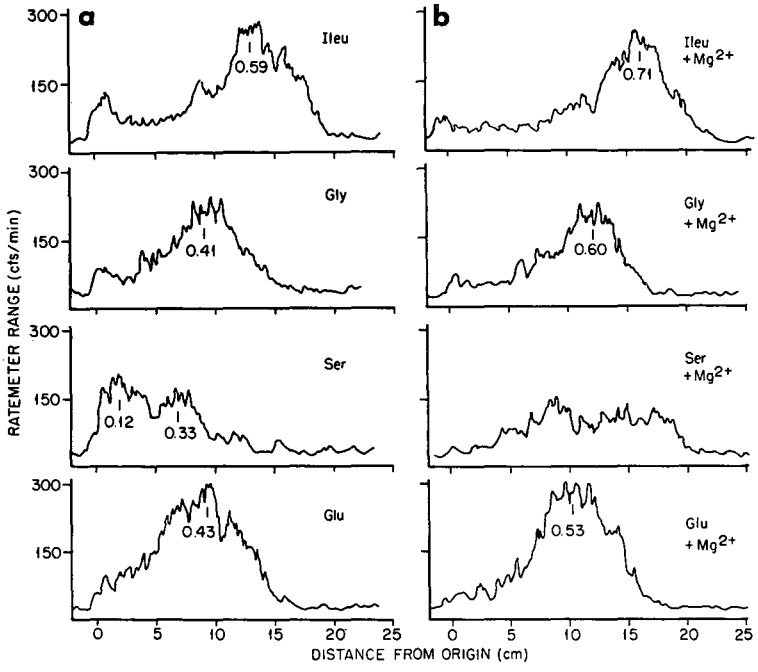


Fig. 1. Chromatography of RNA specific for four different amino acids. Uniformly labeled [^{14}C]-amino acids were esterified to the acceptor-RNA, and 0.05–0.15 ml aliquots of the reaction mixture were applied to DEAE-cellulose paper. (a) The paper was irrigated at 4° with 0.2 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, for 2 h and then with 0.58 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, for 2.5 h. (b) The paper was irrigated for the same time and with the same solution as for Fig. 1a with the addition of 0.01 *M* $MgSO_4$. The positions of the free radioactive amino acids were all at distances greater than 25 cm from the origin. The figure shown under each peak is the mobility relative to GMP.

shown in Fig. 1a. Isoleucyl-RNA moved about 13 cm in 2.5 h in 0.58 *M* NaCl, whereas the presence of 0.01 *M* Mg^{2+} in this developer increased the rate of migration (Fig. 1b). In addition Mg^{2+} also reduced the amount of material that was commonly found at the origin of the chromatogram (*cf.* later sections).

Seryl-RNA separated into two peaks of radioactivity (Fig. 1a) when chromatographed in the absence of magnesium. It is unlikely that this separation is a reflection of the contamination of the radioactive serine with radioactive glycine since the mobility of glycyl-RNA is greater than either peak of seryl-RNA. Furthermore, in the presence of magnesium during chromatography, this seryl-RNA was partially degraded, whereas glycyl-RNA was not (Fig. 1b). Previously, the separation of certain specific amino acyl-RNA's into two fractions has been shown using methylated albumin columns[7] and counter current distribution[13].

Further comparison of the amino acid specific RNA's is shown in Table I. A remarkably similar behavior of the charged RNA's which are specific for a given class of amino acids was noted in that aspartyl- and glutamyl-RNA had similar mobilities, and isoleucyl-, valyl-, and alanyl-RNA were all in the fastest group. It has been reported that on methylated albumin glutamyl- and aspartyl-RNA have very similar elution characteristics[7]. Thus, it would appear that the two RNA's

TABLE I

RELATIVE MOBILITIES OF AMINO ACYL-RNA'S

Following application of samples, the DEAE-cellulose paper was irrigated first with 0.2 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, for 2 h and then the higher NaCl concentration-acetate buffer. R_{GMP} refers to the mobility relative to 5'-GMP applied to the origin at the time the higher concentration of NaCl was initiated.

Exp. No.	*[^{14}C]-amino acyl-RNA*	*Developing conditions*	R_{GMP}
1	ileu	0.58 *M* NaCl	0.55
	lys		0.66
	leu	4°	0.46
	arg		0.43
	his		0.44
	phe		0.32
2	ileu	0.58 *M* NaCl	0.59
	val		0.64
	ala	4°	0.66
	asp		0.38
	glu		0.32
3a	ileu	0.60 *M* NaCl	0.59
	gly		0.41
	ser	4°	0.33, 0.12
	glu		0.43
3b	ileu	0.65 *M* NaCl	0.48
	gly		0.35
	ser	25°	<0.1
	glu		0.32

specific for the two acidic amino acids are themselves rather similar structurally.

In control experiments, the radioactive amino acid was chromatographed under conditions identical to those used for RNA chromatography. The aliphatic amino acids migrated rapidly in 0.2 *M* NaCl, and no radioactivity was detected in the area normally occupied by the RNA; the same was true for lysine, glutamic acid, aspartic acid, serine, threonine. On the other hand, phenylalanine did not move out entirely and a slight trail of radioactivity was found in the RNA areas as well as a small amount (<0.1 %) at the origin; histidine behaved similarly. Since some commercially obtained phenylalanine samples did not behave in this manner, contaminants were probably responsible. Despite this interference, the position of the phenylalanyl- and histidyl-RNA was detectable. With tryptophan, so much radioactivity remained in the RNA region and at the origin that a reliable estimate of the position of the tryptophanyl-RNA could not be made, using the present technic.

A variety of factors affect the migration of amino acyl-RNA on DEAE-cellulose paper. In reporting these experiments, two chromatographic properties of the RNA were studied: (1) the rate of migration under various conditions and (2) the fraction of the total that was found at the origin following the migration of the major portion of the RNA. The latter is due, presumably, to an irreversible binding of the RNA to the paper and may be of significance in explaining the behavior of RNA on substituted celluloses.

Salt concentration

The effect of salt concentration on the rate of leucyl-RNA migration is shown in Fig. 2; the rate is dependent on salt concentration up to 0.7 *M* NaCl where the maximum rate was achieved, as evidenced by the position of the ultraviolet absorbing material as well as the radioactive zone. The change in salt concentration also affected

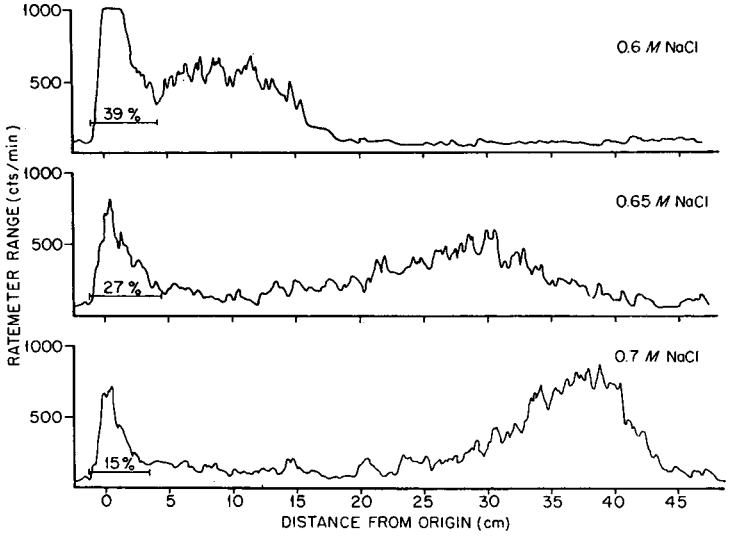


Fig. 2. Effect of salt concentration on [^{14}C]-leucyl RNA chromatography. Samples (0.1 ml) of reaction mixture containing [^{14}C]-leucyl RNA prepared as described in Methods were applied to DEAE-cellulose paper. Irrigation at 25° was with 0.2 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, for, $2^1/_2$ h and then various concentrations of NaCl–0.02 *M* acetate, pH 5.6, for 4 h. The free amino acids had moved off the end of the paper under these conditions. All strips were surveyed at same setting of the count rate meter. Figure at origin indicates the proportion of total radioactivity measured that occurred at the origin area.

the proportion of material that remained at the origin, as much as 39 % of the leucyl-RNA remaining at the lowest salt concentrations. The 15 % at the origin after 0.7 *M* NaCl appeared to be a lower limit since NaCl concentrations up to 5 *M* did not reduce this value.

Examination of non-migrating RNA

If the material that migrated was different from that which remained at the origin, the rechromatography of each should result in single zones. Purified acceptor RNA, with no amino acid attached, was streaked on a DEAE-cellulose paper sheet, moved out in 0.65 *M* NaCl, and the forward and origin zones eluted separately with 1 *M* NaCl. Recovery from the origin zone was poor but sufficient to yield enough RNA to react with [^{14}C]-leucine. Rechromatography of the material from each zone again gave two zones as detected by radioactivity and ultraviolet absorption, one at the origin and one that migrated in 0.65 *M* NaCl. Thus, it appears that the RNA found at the origin after chromatography was not necessarily different from that which migrated but resulted from irreversible adsorption of a portion of the RNA.

Temperature

The experiments immediately preceding were performed at room temperature (22–25°). At 4° there was a decreased rate of migration of leucyl-RNA, as would be expected, and there was a marked decrease in the amount of material left at the origin (Fig. 3). An approximately proportional reduction in migration rate occurred for GMP.

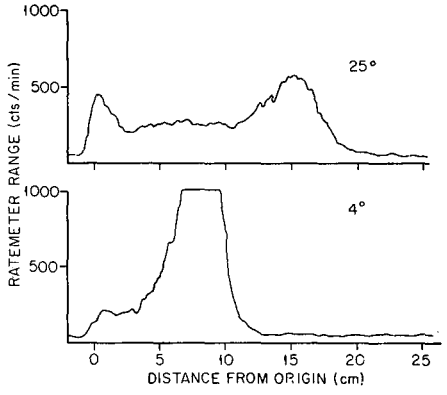


Fig. 3. Effect of temperature on [^{14}C]-leucyl RNA chromatography. Samples were the same as for Fig. 2. One paper remained at 4° before and after application of sample, the other paper was at 25°. Each was irrigated with 0.2 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, for 2 h and then with 0.7 *M* NaCl–0.02 *M* acetate buffer, pH 5.6 for 1.7 h. At 4° the GMP, which was applied to the paper when 0.7 *M* NaCl was initiated, moved 0.58 of the distance attained at 25°. An ultraviolet-absorbing area was observed at the origin of the 25° paper but not of the 4° paper.

Time of contact with the paper

If diminished binding at the origin at 4° was due to a reduction in the rate of formation of the non-ionic bonds, it should be possible to observe an increase in the amount at the origin by extending the time of contact between the RNA and the DEAE-cellulose. Fig. 4 shows that the amount does increase significantly with time, the first few hours resulting in binding of about 10% of the amino acyl-RNA near the origin. It should be noted that in all but the uppermost chromatogram the papers were irrigated with 0.2 *M* NaCl, pH 5.6, for 2 h; the papers then were allowed to hang in the humid atmosphere of the tank until irrigated with 0.7 *M* NaCl. In other experiments, 0.2 *M* NaCl, pH 5.6, was flowing through the paper constantly during the contact period, and similar results were obtained.

In addition to an increase in the binding at the origin, Fig. 4 also shows that the rate of migration of the RNA was reduced by extended time in contact with the paper. It is evident that certain characteristics of RNA undergo alteration in the presence of DEAE-cellulose resulting in more effective adsorption and slower mobility.

One explanation of these phenomena is that the secondary structure of the acceptor-RNA undergoes an alteration, such as the breaking of hydrogen bonds, during contact with DEAE-cellulose. The resulting structure could have a higher affinity for DEAE-cellulose because a greater flexibility in the molecule would result and allow a greater number of attachments between the primary phosphate and the DEAE. In addition to the electrostatic interaction, an increase in the extent of other

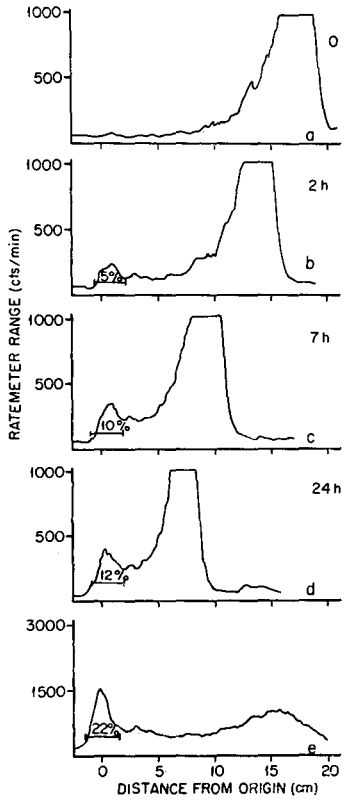


Fig. 4. Effect of time of contact of [^{14}C]-leucyl RNA with DEAE-cellulose. Samples of reaction mixture, 0.05 ml were prepared as in Fig. 2. (a) Paper was irrigated with 0.7 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, for 1 h immediately after application of samples. (b) Paper was irrigated with 0.2 *M* NaCl–0.02 *M* acetate buffer pH 5.6 for 2 h and then with 0.7 *M* NaCl–0.02 *M* acetate buffer, pH 5.6 for 1 h. (c) After irrigation with 0.2 *M* NaCl–0.02 *M* acetate pH 5.6, the paper hung in the chromatography tank for 5 h and then was irrigated with the 0.7 *M* NaCl developer. (d) Paper hung 22 h before irrigation with the higher salt concentration. In 4a-d the chromatography was performed at 4°; in 4e, at 23° and irrigated as in 4b.

attractive forces between the RNA and the cellulose matrix of the paper could also occur.

Effect of urea

Since non-ionic bonds seemed to exist between the RNA and the cellulose and would be largely unaffected by salt solutions, 6 *M* urea was added to the usual developer. The effect of urea was to prevent any amino acyl-RNA from remaining at the origin and to cause 4 amino acyl-RNA's (ileu, gly, ser, glu) to migrate at the same rate as GMP.

Amount of RNA

The amount of RNA applied to the DEAE-paper can be varied at least 20-fold without appreciably changing the migratory behavior. Fig. 5 shows that the positions of the peaks were quite similar whether 0.02 or 0.4 ml of the reaction mixture was applied.

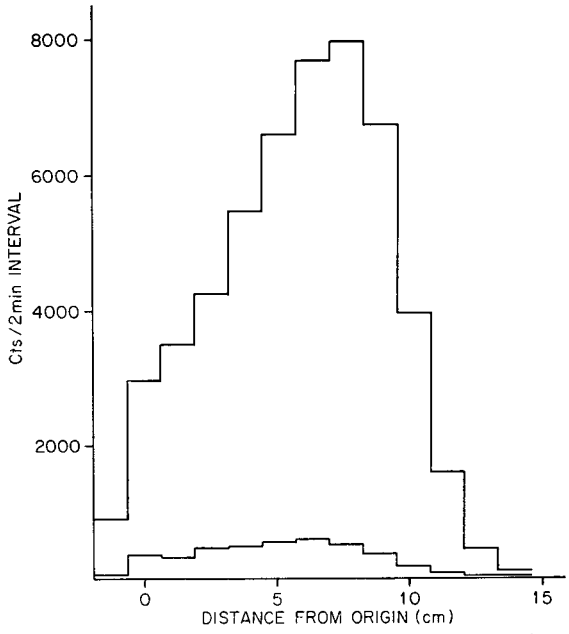


Fig. 5. Chromatography of different amounts of [^{14}C]-leucyl RNA. Samples of a reaction mixture, 0.02 ml and 0.4 ml, were used. The [^{14}C]-leucyl-RNA migrated in 0.58 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, at 4° for $2^1/_2$ h. The data shown are the counts integrated over 2 min intervals, scanning at 25 cm/h through 1 cm apertures.

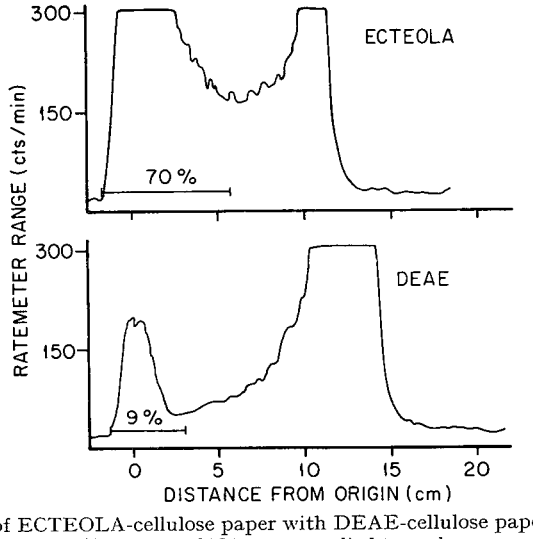


Fig. 6. Comparison of ECTEOLA-cellulose paper with DEAE-cellulose paper. Samples of reaction mixture, 0.05 ml containing [^{14}C]-leucyl RNA, were applied to each paper which had been converted to chloride form as described in Methods. Both papers were irrigated at 24° with 0.2 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, for 4 h and then 1 *M* NaCl–0.02 *M* acetate buffer, pH 5.6, for 1.25 h. The GMP which was applied at the time 1 *M* NaCl was started moved 0.7 as far on ECTEOLA as compared to DEAE paper.

The fraction of RNA irreversibly bound at the origin is independent of the amount of sample applied; *e.g.*, samples of 0.02, 0.05 and 0.1 ml of isoleucyl-RNA were chromatographed and the amount remaining at the origin was 8.5, 4.8 and 5.9%, respectively.

ECTEOLA-cellulose paper

Because ECTEOLA-cellulose is also commonly used for chromatography of nucleic acids, it was compared with DEAE-cellulose, both materials being used as paper sheets. Fig. 6 demonstrates that, under the same conditions, leucyl-RNA behaves quite differently on ECTEOLA-cellulose. The material at the origin presumably was due, in part at least, to the anomalous behavior described for DEAE paper, the extent being predictably minimal on the latter because of the 1 *M* NaCl. No further exploration of the ECTEOLA paper was made. Experience on ECTEOLA-cellulose columns indicated that a strong binding of RNA occurred that was broken only by partially degrading the RNA[14].

DISCUSSION

Among the technics available for the fractionation of amino acyl acceptor-RNA (see Introduction), the DEAE-cellulose paper method is simple, inexpensive, and rapid. Furthermore, it can be adapted to a preparative scale; an 18-cm wide sheet has been used to chromatograph 3 mg of acceptor RNA and 100% recovery of the acceptor activity for 4 amino acids was obtained by eluting the developed chromatogram with 1 *M* NaCl–6 *M* urea (JACOBSON AND NISHIMURA, unpublished data). Combination of DEAE-cellulose paper chromatography and other technics, such as methylated albumin chromatography, could prove productive in purifying certain acceptor RNA's since the relative mobilities of several of the amino acyl-RNA's are different on the two media. The effect of urea was to abolish the resolution of specific acceptor RNA's on DEAE-cellulose just as it was to cause oligodeoxyribonucleotides to migrate according to molecular size[15].

Some insight into the nature of the interaction between acceptor RNA and DEAE-cellulose is given by these experiments. There are apparently two phenomena to be explained: (1) the nature of the binding of various amounts of the acceptor RNA at the origin, and (2) the alteration in the RNA which results in the slower migration rate illustrated in Fig. 4. With regard to the RNA found at the origin, two types of forces are operative since the amount varied between 15 and 40% with salt concentration but could not be reduced below this lower amount by further increasing the salt concentration. This indicates that non-ionic interactions occur between the RNA and the DEAE-cellulose resulting in attachments that cannot be dissociated by salt. Since these bonds form slowly but at a discernible rate, in terms of hours at 4° (Fig. 4), there are apparently many such bonds per RNA molecule. When only a few non-ionic bonds exist, they may not be sufficiently stable to prevent the RNA from migrating at higher salt concentration but they may be able to supplement the ionic interaction of the acceptor RNA and DEAE-cellulose at lower salt concentrations and cause more RNA to be retained at the origin (Fig. 2).

Such forces would not be apparent in the chromatography of mononucleotides and the smaller oligonucleotides since only a very few such bonds could form per molecule. On the other hand, for a polymer consisting of 90–100 nucleotides there would

be the possibility for multiple attachments resulting in a strong interaction not reversible by high molarities of salt.

Other laboratories have reported irreversible attachment of various RNA's to anion exchange cellulose usually evidenced by incomplete recovery of the sample. GOLDTHWAIT AND KERR[14] found ribosomal RNA was bound by ECTEOLA-cellulose so strongly that the alkaline conditions employed for elution resulted in material of diminished sedimentation coefficients.

The nature of the forces resulting in irreversible binding of acceptor RNA at the origin can be surmised, speculating from the effects of urea, magnesium ion, and temperature. It is unlikely that hydrogen bonds are involved since they would be expected to be less stable at 25° than at 4°, whereas greater binding was found at the higher temperature. Urea, although often considered to break hydrogen bonds, is effective in disrupting hydrophobic bonds of DNA resulting in a denaturation of the molecule[16]. Therefore, the fact that urea completely removes the acceptor RNA from the origin position of the DEAE-cellulose does not necessarily indicate hydrogen bonding but that hydrophobic bonds may exist between the RNA and the paper. The fact that magnesium ion reduces the amount of RNA at the origin is not inconsistent with an hydrophobic bond interpretation since it is known that magnesium ion causes a configurational change in acceptor RNA to a more helical form[17]. Such a configuration would probably reduce the hydrophobic bonds possible by making the purine and pyrimidine rings less available.

The second phenomenon, the reduction in the rate of acceptor RNA migration as a function of contact time with the DEAE-cellulose, may be a reflection of distortion of the RNA molecule. This distortion may arise from the electrostatic interaction between acceptor RNA and the DEAE-cellulose, or a combination of electrostatic and non-ionic forces, which might disorganize some of the secondary structure of the RNA and result in a more flexible molecule. In a more extended, flexible state the RNA phosphate groups may be able to engage in more interactions per molecule with the positively charged secondary amine groups of the paper, thus resulting in a slower mobility. The retarded mobility resulting from prolonged contact with the adsorbent apparently is not due to the same phenomenon responsible for the binding of the RNA at the origin. While a parallel exists in that both effects progress with time, the binding at the origin never accounts for all the RNA that might be expected if the retardation was proceeding progressively. Rather, the two phenomena each seem to reach a limit and thus do not appear to be due to common mechanism.

SUMMARY

Conditions for the chromatography of amino acyl-acceptor RNA on paper sheets of DEAE-cellulose have been explored further. Amino acid-specific RNA's can be separated using this type of chromatography. It was found that significant amounts of RNA are bound to the paper so that (1) salt solutions were ineffective eluents of RNA, and (2) urea combined with salt was effective. Results suggest that non-electrostatic forces play a significant role in the adsorption of RNA to substituted cellulose.

REFERENCES

[1] A. BENDICH AND H. S. ROSENKRANZ, *Arch. Biochem. Biophys.*, 94 (1961) 417.
[2] K. NISHIYAMA, T. OKAMOTO, I. WATANABE AND M. TAKANAMI, *Biochim. Biophys. Acta*, 47 (1961) 193.
[3] E. J. OFENGAND J. DIECKMANN AND P. BERG, *J. Biol. Chem.*, 236 (1961) 1741.
[4] K. C. SMITH, E. NORDES AND R. S. SCHWEET, *Biochim. Biophys. Acta*, 33 (1959) 286.
[5] R. W. HOLLEY AND S. H. MERRILL, *J. Am. Chem. Soc.*, 81 (1959) 753.
[6] H. G. ZACHAU, M. TAKEDA, W. B. LAWSON AND M. SCHWEIGER, *Biochim. Biophys. Acta*, 53 (1961) 221.
[7] N. SUEOKA AND T. YAMANE, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1454.
[8] G. HARTMANN AND U. COY, *Biochim. Biophys. Acta*, 47 (1961) 612.
[9] F. LIPMANN, W. C. HULSMANN, G. HARTMANN, H. G. BOMAN AND G. ACS, *J. Cellular Comp. Physiol.*, 54, Suppl. 1 (1959) 75.
[10] K. B. JACOBSON AND S. NISHIMURA, *Biochim. Biophys. Acta*, 68 (1963) 490.
[11] J. PREISS, M. DIECKMANN AND P. BERG, *J. Biol. Chem.*, 236 (1961) 1748.
[12] S. NISHIMURA AND G. D. NOVELLI, *Biochim. Biophys. Acta*, (1964) in press.
[13] B. WEISBLUM, S. BENZER AND R. W. HOLLEY, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1449.
[14] D. A. GOLDTHWAIT AND D. S. KERR, *Biochim. Biophys. Acta*, 61 (1962) 930.
[15] R. V. TOMLINSON AND G. M. TENER, *J. Am. Chem. Soc.*, 84 (1962) 2644.
[16] L. LEVINE, J. A. GORDON AND W. P. JENCKS, *Biochemistry*, 2 (1963) 168.
[17] S. NISHIMURA AND G. D. NOVELLI, *Biochem. Biophys. Res. Commun.*, 11 (1963) 161.

A SIMPLE DEVICE FOR CONTINUOUS ELUTION IN FILM CHROMATOGRAPHY

E. V. TRUTER

Textile Chemistry Laboratory, The University, Leeds (Great Britain)

(Received July 17th, 1963)

INTRODUCTION

In spite of the excellent results that can be obtained by normal film chromatography, there are situations in which a single development of a chromatogram does not give satisfactory resolution of the components of a mixture. To improve the resolution, two equivalent methods are available. In the first, the process of multiple development, the chromatogram is developed in the normal way. When the solvent reaches a pre-determined level on the film, the chromatoplate (or strip) is withdrawn from the developing tank, and after the solvent has evaporated from the film, the chromatogram is again developed in the normal manner. The process may be repeated as often as is necessary to obtain a satisfactory result. Equations for determining the optimum number of developments have been given by TRUTER[1].

The technique of multiple development is tedious and less convenient to operate than the alternative process, namely continuous development. Unfortunately, to subject a film chromatogram to continuous development, special apparatus is required. In those pieces of equipment that have been described hitherto, the direction of solvent-flow is abnormal. Film chromatograms are normally developed by the ascending-solvent technique. In the devices designed by STANLEY AND VANNIER[2], by MISTRYUKOV[3] and by REISERT AND SCHUMACHER[4] the solvent-flow is downward, and in the devices described by MOTTIER[5] and by BRENNER AND NIEDERWIESER[6] it is horizontal. As a consequence of the abnormal direction of solvent-flow, special arrangements are required for feeding the solvent on to the film.

To maintain a continuous flow of liquid through the film, the solvent must be removed when it reaches the further limit of the adsorbent. In the descending-development technique the solvent is allowed to drip off the chromatogram whereas in the horizontal-development technique the solvent is allowed to evaporate when it reaches the far edge of the adsorbent. Hitherto, no simple and satisfactory method for continuous development by the ascending-solvent technique has been described. If a chromatogram is developed in an open tank, the results are unsatisfactory because the solvent evaporates from the entire surface of the chromatogram; for satisfactory results, it is absolutely essential to limit evaporation of the solvent to a strip of adsorbent which is not part of the working area. In the apparatus described here, continuous development is obtained by allowing the solvent to ascend the film which projects through a slot in the lid of the tank. The dimensions of the slot are such as to restrict evaporation to that part of the adsorbent which is outside the tank.

Besides permitting continuous development to be carried out very simply, the slotted lid can also be employed as an auxiliary in increasing the resolution in a single-stage development. In general, the smaller the spot of sample at the origin, the better will be the resolution. The slotted lid may be used for converting a relatively large spot of sample placed at the origin into a line about 0.5 mm wide at the opposite side of the film.

EXPERIMENTAL

Apparatus

The process described here requires a developing tank from which the strip or plate will project by 1–2 cm when placed vertically in it. Other dimensions of the tank should be the minimum required to hold the plate. The lid is the important feature. For the development of chromatostrips, the lid is in two parts; one is a specially shaped piece of brass and the other is merely a glass cover for the remainder of the tank. For standard plates it is more convenient to use a machined brass strip and two glass plates as the lid. The essential features of the design of the brass strip are shown in Fig. 1. The dimensions of the brass strip which are not shown must be adjusted to

Fig. 1. Plan of the machined brass strip which is 6 mm thick. The hatched area represents the section of the chromatoplate.

suit the developing tank and the glass plate available so that, when the apparatus is assembled, the chromatoplate is effectively sealed all round except for the small gap facing the adsorbent film. When the brass is 6 mm thick, a gap of about 1 mm between the adsorbent surface and the recess in the brass strip is satisfactory; if thinner sheets of brass are used, the gap must be correspondingly smaller. During development of the chromatogram, the solvent climbs up the film and when it reaches the exposed part of the adsorbent outside the tank, it evaporates, and so continuous development of a vertical chromatogram is achieved.

Methods

A solution of a commercial dye, Oil Red OS, in methylene chloride was spotted on to a chromatoplate which had been prepared in the conventional manner using silicagel G (film thickness: 250 μm). The charged plate was then placed in the tank with the adsorbent-coated surface of the glass facing the brass strip and "clamped" by closing up to the other portions of the lid. Carbon tetrachloride was used as the developing solvent and the sides of the tank were lined with filter-paper soaked in solvent.

Formation of a hair-line origin was carried out as follows. A line sample of a solution of Oil Red OS was placed on the adsorbent in the usual way, the charged plate was placed in the developing tank, the slotted-lid was assembled and the chromatogram was "developed" in chloroform–methanol (10:1). After about 1 h the dye sample had been transferred to the opposite end of the chromatoplate where it formed a line about 0.3 mm in width. The plate was then withdrawn from the tank and after the solvent had evaporated from the film, the adsorbent was reactivated by heating

the chromatoplate to 110° for 5 min. Subsequently, the chromatogram was developed from the hair-line origin, using carbon tetrachloride as the solvent, by the continuous technique already described (Fig. 2); the hair-line origin is still clearly marked by the most polar fraction.

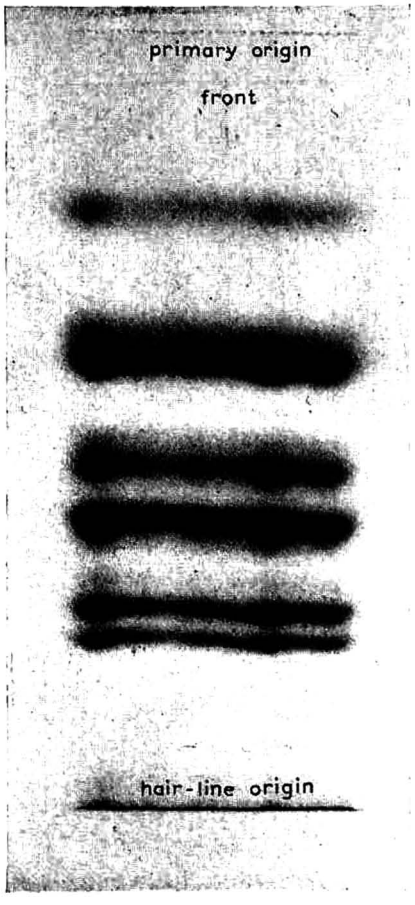


Fig. 2. One-dimensional continuous development of Oil Red OS from a hair-line origin. Adsorbent: silicagel G. Solvent: carbon tetrachloride. Time: 18 h. Distance from origin to front: 15 cm.

The chromatogram shown in Fig. 3 was prepared in a similar manner. After development in the first direction in carbon tetrachloride, the sample was compressed into a second hair-line origin at right angles to the first origin by development in chloroform–methanol (10:1). Subsequent treatment of the chromatogram was exactly as described above.

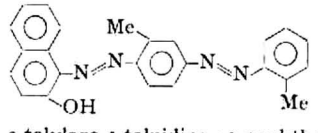
RESULTS AND DISCUSSION

For the formation of a hair-line origin a good solvent, which carries the sample with or near the solvent front, is required. When the solvent level reaches the upper edge of the slot, it evaporates and the sample is gradually deposited in the form of a line at the edge of the solvent front. With colourless samples it is important to ensure that all

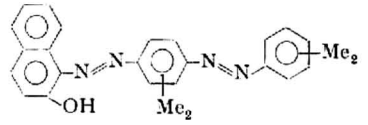
Fig. 3. Two-dimensional continuous development of Oil Red OS. Adsorbent: silicagel G. Solvent: carbon tetrachloride. Time: 16 h in each direction. Distance from origin to front: 16 cm.

the material has reached the front; no difficulty is encountered if the process is allowed to continue for longer than the minimum time. If a solvent which can de-activate the adsorbent has been used to produce the hair-line origin, *e.g.*, one containing methanol, the adsorbent should be reactivated by a brief thermal treatment. Under normal laboratory conditions hydroxylic solvents alone are not suitable for preparing a hair-line origin because they do not evaporate quickly enough; they form rather irregular fronts *above* the level of the slot.

In two-dimensional work the hair-line origin may be used for either or both developments. Fig. 3 shows an overloaded (total load: 2.4 mg) two-dimensional chromatogram in which the hair-line origin was used for both directions. The test sample, Oil Red OS, is a mixture of:



4-*o*-tolylazo-*o*-toluidine→2-naphthol and



xylylazo-xylidine→2-naphthol

As all the positional isomers of xylene are present in the starting material, the product would be expected to be a complex mixture of isomers. Fig. 3 shows that the mixture has been resolved into fourteen fractions and on the original chromatogram an addi-

tional four faint spots could be detected. Normal chromatography of the dye, using either benzene or methylene chloride as the developing solvent, failed to resolve the mixture into more than two fractions.

SUMMARY

A simple, inexpensive slotted lid which enables a film chromatogram to be developed continuously, and its application for transferring a sample to a hair-line origin, is described.

REFERENCES

1 E. V. TRUTER, *Thin Film Chromatography*, Cleaver-Hume, London, 1963, p. 63.
2 W. L. STANLEY AND S. H. VANNIER, *J. Assoc. Offic. Agric. Chem.*, 40 (1957) 582.
3 E. A. MISTRYUKOV, *J. Chromatog.*, 9 (1962) 311.
4 P. M. REISERT AND D. SCHUMACHER, *Experientia*, 19 (1963) 84.
5 M. MOTTIER, *Mittlg. Lebensmit. Hyg.*, 49 (1958) 454.
6 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 17 (1961) 237.

RESOLUTION OF *n*-ACIDS AND *n*-ALCOHOLS BY "ADSORPTION" CHROMATOGRAPHY ON KIESELGUR FILMS

S. JEAN PURDY AND E. V. TRUTER
Textile Chemistry Laboratory,
The University, Leeds (Great Britain)

(Received July 26th, 1963)

INTRODUCTION

It is generally believed that the resolution of mixtures of aliphatic compounds belonging to a homologous series is not possible by normal adsorption chromatography if the compounds contain more than about ten carbon atoms each. Resolution of mixtures of this type, *e.g.*, *n*-alcohols or *n*-acids is usually performed by reversed-phase partition chromatography[1]. If adsorption chromatography could be adapted to give a degree of resolution comparable with that obtainable by reversed-phase partition chromatography, the former would be preferable because it is more rapid and much simpler.

Adsorption chromatography is normally carried out on silicagel or alumina, and experience has shown that alcohols and acids can be chromatographed only by using relatively polar solvents. Under these circumstances, the difference between the eluting effect of the solvent and the restraining effect of the adsorbent is the same for all higher members of a homologous series, so that the mixture cannot be resolved into its components.

There are, nevertheless, small differences in the polar/non-polar balance between members of a homologous series. We have explored the possibility of magnifying the effect of these minor differences by simultaneously decreasing both the restraining power of the adsorbent and the eluting power of the solvent. The results showed that the hitherto unexpected resolution of homologous, even-numbered alcohols and acids containing more than ten carbon atoms, was possible by "adsorption" chromatography on kieselgur G, using cyclohexane as the developing solvent.

EXPERIMENTAL

Kieselgur G and water (30 g/60 ml) were thoroughly mixed and spread over six glass plates (15 × 20 cm) using a device similar to that described by STAHL[2]. After the films had been allowed to dry on the bench for 3 h they were ready for use. Samples of the *n*-alcohols and the *n*-acids were made into 0.5 % and 0.25 % solutions (respectively) in ether, and 1 μl aliquots were used for chromatography; the mixtures contained 1 μg of each component. Development of the chromatograms with cyclohexane, in tanks lined with filterpaper soaked in the solvent, for a distance of 10 cm, required about 35 min. To render the alcohols visible, the solvent-free film was sprayed

with concentrated sulphuric acid and the chromatoplate was heated to 160° for 10 min. The chromogenic reagent for the acids was 0.5 % bromocresol green in ethanol, adjusted to pH 6.

RESULTS AND DISCUSSION

Fig. 1 shows that it is possible to resolve and to identify a mixture of all the even-numbered, aliphatic alcohols from *n*-decanol to *n*-tetracosanol. In addition, *n*-octanol and *n*-hexacosanol may also be distinguished, but with a lower degree of certainty.

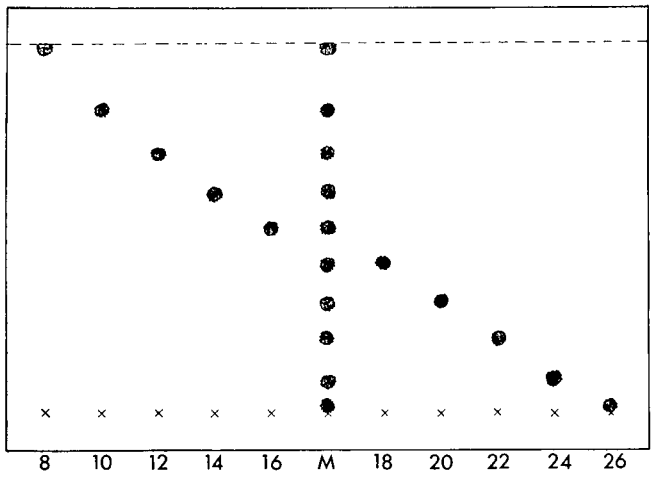


Fig. 1. Resolution of the *n*-alcohols by "adsorption" chromatography. The numerals indicate the number of carbon atoms in each alcohol. M is a mixture of all the test samples. (Substrate: kieselgur G. Solvent: cyclohexane. Chromogenic reagent: sulphuric acid).

Fig. 2 shows that the identification of the even-numbered acids containing between eight and fourteen carbon atoms presents no difficulty. Acids containing between sixteen and twenty-two carbon atoms per molecule are less well separated from one another. Although the R_F values are appreciably different, the resolution is not sufficient for an unequivocal identification if the length of the chromatogram is only 10 cm. Development of the chromatogram for a longer distance will result in a better resolution and a correspondingly higher degree of certainty in the identification.

The tailing effect which is observed only with the acids, is very dependent on the load. If the load is progressively increased beyond 2.5 μg per acid, tailing rapidly becomes bad enough to spoil the chromatogram. If a small amount of acetic acid (0.5 %) is added to the developing solvent to prevent tailing, the mobilities of all the acids are greatly increased and the differences between their R_F values are substantially diminished.

Not only do the acids exhibit an undesirable tendency to tail but the range of molecular chain-lengths which can be resolved is rather restricted. Nevertheless, a useful degree of resolution is obtainable by chromatography on kieselgur G, particularly if the chromatogram is developed for a distance exceeding 10 cm. Resolution of the alcohols is sufficiently good to be comparable with that obtainable by the slightly more discriminating but much more time-consuming process of reversed-phase partition chromatography.

It was observed that the R_F values of the alcohols and the acids varied slightly

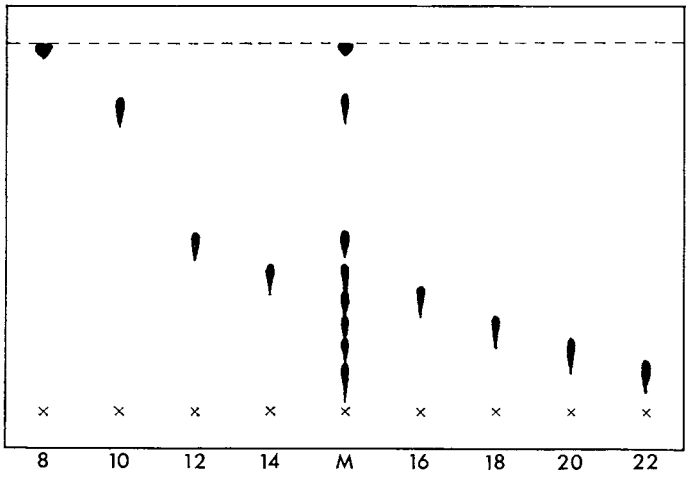


Fig. 2. Resolution of the *n*-acids by "adsorption" chromatography. The numerals indicate the number of carbon atoms in each acid. M is a mixture of all the test samples. (Substrate: kieselgur G. Solvent: cyclohexane. Chromogenic reagent: bromocresol green).

according to the period for which the kieselgur films had been allowed to dry. On films that had been dried by treatment at 110° for 30 min the R_F values were low and the resolution was poor. These observations suggest that partition, between the residual water in the kieselgur and the moving phase, makes an important contribution to the degree of resolution.

By continuous development, it was possible to obtain satisfactory resolution of a mixture of all the even-numbered fatty acids from *n*-decanoic to *n*-docosanoic on kieselgur G films which had been baked for 30 min at 110°. As before, cyclohexane was the developing solvent, but the development was performed in a tank fitted with the slotted lid described by TRUTER[3]. The time required for development was 4 h and the distance from the front to the origin was 14 cm.

ACKNOWLEDGEMENT

Part of the cost of this work was defrayed by a grant from the Agricultural Research Council.

SUMMARY

It is shown that "adsorption" chromatography on films of kieselgur G, using cyclohexane as the developing solvent, will resolve mixtures of the even-numbered, *n*-alcohols containing between ten and twenty-six carbon atoms per molecule within a distance of 10 cm. Resolution of similar mixtures of the *n*-acids under the same conditions is rather less satisfactory, but it is entirely satisfactory if the chromatogram is submitted to continuous development.

REFERENCES

1 H. P. KAUFMANN AND Z. MAKUS, *Fette, Seifen, Anstrichmittel*, 62 (1960) 1014.
2 E. STAHL, *Chemiker-Zeitung*, 82 (1958) 323.
3 E. V. TRUTER, *J. Chromatog.*, 14 (1964) 57.

IDENTIFICATION OF N-NITROSO- AND NITRODIPHENYLAMINES BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY*

STANLEY K. YASUDA

University of California, Los Alamos Scientific Laboratory, Los Alamos, N. M. (U.S.A.)

(Received August 5th, 1963)

INTRODUCTION

Diphenylamine (DPA) has been used extensively as a stabilizer in nitrocellulose (NC) containing explosives and propellants. Its primary function is to react with the oxides of nitrogen formed by the slow decomposition of the NC, thereby being converted into the corresponding N-nitroso- and nitro- derivatives.

The complexity of diphenylamine products is well known[1], and very few identification methods are reported in the literature. HANSSON AND ALM[2] describe a one-dimensional thin layer chromatographic (TLC) method for the identification of eight derivatives of diphenylamine; however, several important derivatives, *e.g.*, N-nitroso-dinitro- and trinitrodiphenylamine, are not included in the study. This is understandable, since authentic DPA derivative samples are difficult to obtain.

The excellent work of SCHROEDER *et al.*[3] utilized column chromatography to identify and estimate N-nitroso- and nitrodiphenylamine products in smokeless powder; however, the efficiency of separation and method of locating the products proved unsatisfactory for our use. Other quantitative methods were not applicable for identification work, and were eliminated from further consideration.

This article presents a two-dimensional TLC method to separate and identify nineteen N-nitroso- and nitrodiphenylamines. A spray reagent capable of detecting a 0.5 to 1 μg quantity of each product is described. Samples of PBX-9404, a plastic bonded explosive, and reaction products of DPA and 2-nitroDPA with nitrogen tetroxide were examined by the method developed.

REAGENTS AND EQUIPMENT

Applicator, chromatojar, silica gel G, and glass plates (200 × 200 mm), purchased from Brinkmann Instruments, Inc.

Zinc metal dust, AR grade, from Mallinckrodt Chemical Works.

All solvents were reagent grade. Petroleum ether from Merck and Co., Inc., with a boiling range of 30–60° was used.

The spray reagent consisted of 0.25% *p*-diethylaminobenzaldehyde (*p*-DEAB) and 0.25 *N* HCl in absolute ethanol. Concentrated HCl was used to prepare the reagent.

* Work done under the auspices of the U.S. Atomic Energy Commission.

An Agla micrometer syringe or capillary melting point tube was used as an applicator.

EXPERIMENTAL

Preparation of thin layer

Modification of STAHL's technique for thin layer preparation was necessary to incorporate zinc dust directly into the silica gel G, a procedure previously described[4]. A slurry mixture of 30 g of silica gel G and 3 g of zinc dust in 65 ml of rapidly stirred water was prepared. Coating was accomplished by pulling the applicator with a Bodine speed reductor motor at a constant speed over five glass plates. The silica gel G/Zn plates were activated at 110° for 1 to 2 before use.

R_G measurements

Since nineteen N-nitroso- and nitrodiphenylamines were included in this work, it was apparent that complete separation could not be accomplished by a one-dimensional technique; therefore, solvents were screened, using a simple DPA-derivative mixture as the sample on activated silica gel G/Zn plates. Based upon separation efficiency, several solvents were selected and tested with more complex mixtures. The two most promising solvents were, (1) 2:99:99 acetone–benzene–petroleum ether (solvent I), and (2) 20:80 ethyl acetate–petroleum ether (solvent II). Each authentic sample of N-nitroso- and nitrodiphenylamine was tested in solvents I and II. The measured R_G values are plotted graphically in Fig. 1, using DPA as the reference compound.

Procedure

Finely powdered explosives samples, *e.g.*, PBX-9404 (HMX/NC/tris(2-chloroethyl)-phosphate/DPA), were extracted for 2 h at room temperature, using 25 ml of methylene chloride per 0.4 g of sample. Use of a wrist-action shaker is suggested. The solvent was decanted and filtered, an additional 25 ml of solvent was added, and the extraction repeated for 1.5 h. The combined extract was placed under mild vacuum to strip off the solvent. About 0.2 ml of acetone was used to bissolve the residue.

Samples of the DPA/N_2O_4 and 2-nitroDPA/N_2O_4 reaction products were dissolved directly in acetone.

The acetone solution, containing about 100–400 mg of DPA derivatives, was transferred to a point approximately 1.5 in. from the lower right-hand corner of an activated silica gel G/Zn plate. Transfer was accomplished by using an Agla micrometer syringe or melting point capillary. The diameter of the applied spot was kept at or below 0.5 in.

The plate was chromatographed in 200–250 ml of solvent I for 1 h, exposed to the atmosphere for several minutes to evaporate the solvent, and rechromatographed at 90° to the original solvent flow direction in an equal volume of solvent II for an additional hour.

The separated N-nitroso- and nitrodiphenylamine products were located by spraying the plate uniformly with *p*-DEAB reagent. The plate was heated with a hair dryer, particularly in the DPA/2-nitroDPA region, to intensify the colored spots.

RESULTS AND DISCUSSION

Fig. 1 indicates that nineteen of the twenty N-nitroso- and nitrodiphenylamines

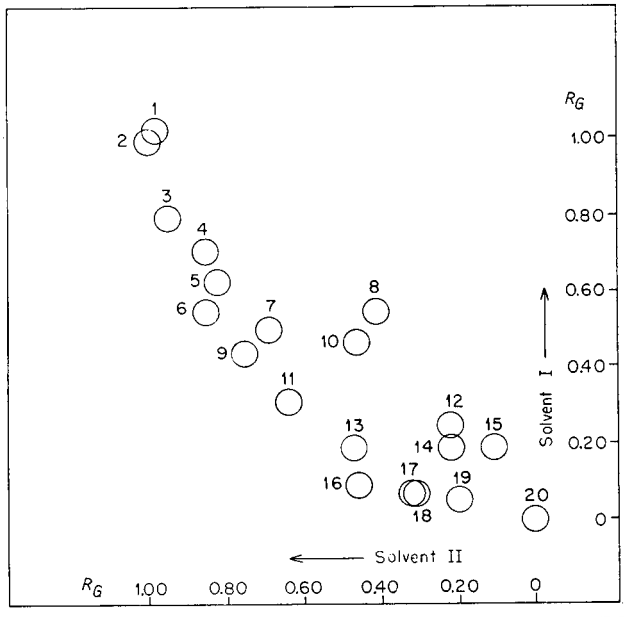


Fig. 1. R_G measurements of N-nitroso- and nitrodiphenylamines. (1) 2-nitroDPA; (2) DPA; (3) N-nitrosoDPA; (4) N-nitroso-4-nitroDPA; (5) 2,4-dinitroDPA; (6) 2,4,6-trinitroDPA; (7) 2,4′-dinitroDPA; (8) 2,2′-dinitroDPA; (9) N-nitroso-4,4′-dinitroDPA; (10) N-nitroso-2-nitroDPA; (11) 4-nitroDPA; (12) N-nitroso-2,4′-dinitroDPA; (13) 2,4,4′-trinitroDPA; (14) 2,2′,4-trinitroDPA; (15) N-nitroso-2,2′-dinitroDPA; (16) 4-nitrosoDPA; (17) 2,2′,4,4′-tetranitroDPA; (18) 2′,2′,4,4′,6-pentanitroDPA; (19) 4,4′-dinitroDPA; (20) 2,2′,4,4′,6,6′-hexanitroDPA.

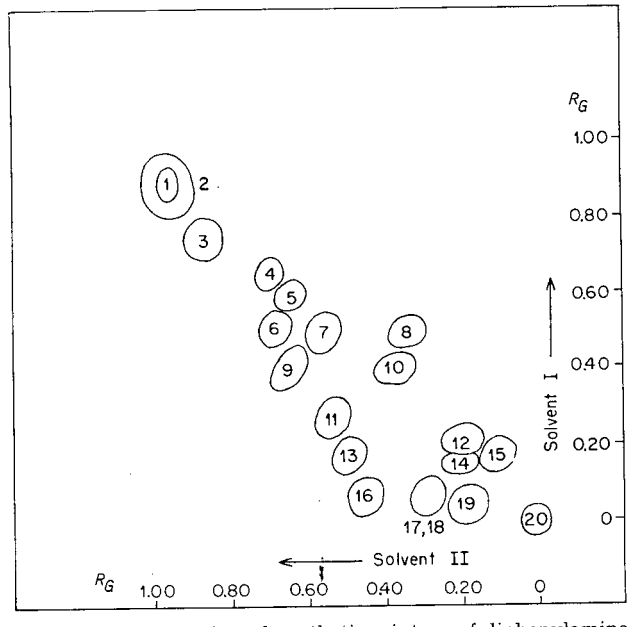


Fig. 2. Two-dimensional separation of synthetic mixture of diphenylamine derivatives.

could be partially or completely separated for identification. Only the tetranitro- and pentanitrodiphenylamines are not separable. Attempts to resolve these spots caused poor separation of the other diphenylamines.

To verify the R_G measurements, a synthetic mixture of twenty components was chromatographed. Figure 2 is a tracing of the developed plate. Close resemblance between Figs. 1 and 2 is noted, although in general the spots in Fig. 2 are displaced. The tracing is, however, adequate for identification work. It is likely that the spot displacement observed in Fig. 2 is due to the influence of each component on the migration rate of the others.

From Fig. 2 it can also be seen that DPA and 2-nitroDPA overlap more than predicted by Fig. 1; however, 2-nitroDPA is a self indicator and can be detected on the unsprayed plate. Furthermore, after spraying and heating, the color intensity of the 2-nitroDPA spot diminishes; an intense yellow to green spot (depending upon the concentration of DPA) develops at the location of the DPA fraction. By using all of the properties of the system, R_G measurement, color, and developed color, it is possible to identify all of the DPA derivatives.

As part of a study on the capacity of various stabilizers for reaction with N_2O_4, samples of the reaction products of DPA and 2-nitroDPA with N_2O_4 and extracts from samples of PBX-9404 were analyzed by the described procedure. Based upon the relative sizes and intensities of the developed spots, N-nitroso- and nitrodiphenylamines found in the samples are listed in Table I in the approximate order of decreasing concentration.

The complexity of the DPA derivatives is evident. Differences between the present results and those reported by HANSSON AND ALM[2] can probably be attributed to the composition of the original sample. For example, the tris(2-chloroethyl)phosphate in the PBX-9404 increases the rate of formation of N-nitroso- and nitrodiphenylamine products as compared with aged DPA/NC mixtures.

TABLE I

IDENTIFIED DIPHENYLAMINE PRODUCTS IN VARIOUS SAMPLES

Sample	*Derivatives found*
PBX-9404-03	N-nitrosoDPA $\gg$ DPA = 2-nitroDPA > 4-nitroDPA > N-nitroso-4-nitroDPA
PBX-9404-03, heated at 60° for 15 weeks	2,4′-dinitroDPA $\geq$ N-nitrosoDPA > N-nitroso-4-nitroDPA = 4-nitroDPA = 2,4,4′-trinitroDPA = 4,4′-dinitroDPA > 2,2′-dinitroDPA > N-nitroso-4,4′-dinitroDPA > N-nitroso-2,2′-dinitroDPA = 2,2′,4-trinitroDPA > 2-nitroDPA $\geq$ tetranitro- or pentanitroDPA $\geq$ 2,4-dinitroDPA

(continued on p. 69)

TABLE I (*continued*)

Sample	*Derivatives found*
PBX-9404, heated at 60° for 24 weeks	2,4′-dinitroDPA > 2,2′-dinitroDPA = 2,4,4′-trinitroDPA > N-nitroso-4-nitroDPA = N-nitrosoDPA ≥ N-nitroso-4,4′-dinitroDPA = 4-nitroDPA = 4,4′-dinitroDPA > N-nitroso-2,2′-dinitroDPA > 2,2′,4-trinitroDPA > 2,4-dinitroDPA = 2-nitroDPA ≥ tetranitro- or pentanitroDPA
DPA/N_2O_4 reaction product	N-nitroso-4,4′-dinitroDPA > 4,4′-dinitroDPA = N-nitroso-2,4′-dinitroDPA > 2,4,4′-trinitroDPA > 2,2′-dinitroDPA > 2,2′,4-trinitroDPA > 2,4′-dinitroDPA = hexanitroDPA > N-nitroso-4-nitroDPA ≥ 2,4-dinitroDPA > N-nitroso-2,2′-dinitroDPA > traces of N-nitrosoDPA, 2-nitroDPA, DPA, tetra- or pentanitroDPA, and two unknowns.
2-nitroDPA/N_2O_4 reaction product*	N-nitroso-2,4′-dinitroDPA ⩾ N-nitroso-2,2′-dinitroDPA ≥ 2,4′-dinitroDPA = N-nitroso-2-nitroDPA = 2,2′,4-trinitroDPA = 2,4,4′-trinitroDPA > 2,2′-dinitroDPA = 2,4-dinitroDPA = hexanitroDPA > traces of tetra- or pentanitroDPA, 4,4′-dinitroDPA, N-nitroso-4-nitroDPA, 4-nitroDPA, and two unknowns.

* 4-nitroDPA was present in the 2-nitroDPA as an impurity.

Reaction conditions used in testing the pure stabilizers, DPA and 2-nitroDPA, were designed to drive the reactions to their practical completion. Note, however, the complexity of the products after the stabilizer capacity of the compounds has been exceeded.

The extraction of DPA and its derivatives from explosives is carried out at ambient temperature to minimize the decomposition of the less stable DPA derivatives. For example, it was found that N-nitroso-4,4′-dinitrodiphenylamine tends to denitrosate and revert to 4,4′-dinitrodiphenylamine. Extractions requiring heating should be avoided.

Authentic samples of N-nitroso- and nitrodiphenylamines were difficult or

extremely expensive to purchase; therefore, most of the compounds tested were prepared in this laboratory. The trinitro-, tetranitro-, and pentanitro-diphenylamines were isolated from nitration products. The nitroso-nitrodiphenylamines were prepared by nitrosating the corresponding nitro- compounds. In all cases, purification was carried out on TLC plates. The purified product was characterized by its melting point, infrared spectrum, and elemental analysis.

The spray reagent, *p*-DEAB, can detect minute quantities of diphenylamine derivatives. For each compound included in this work, 1 μg or less could easily be detected.

ACKNOWLEDGEMENTS

The author is indebted to H. E. UNGNADE for preparation of certain nitrodiphenylamines, and to M. J. NARANJO for elemental analyses.

SUMMARY

A two-dimensional thin-layer chromatographic method, capable of separating and identifying nineteen N-nitroso- and nitrodiphenylamines, is described. The method is applied to the analysis of diphenylamine/N_2O_4 and 2-nitroDPA/N_2O_4 reaction products and the stabilizer fraction of a plastic bonded explosive. A spray reagent, *p*-diethylaminobenzaldehyde, used in conjunction with silica gel G/Zn plates, offers a sensitive detection method for each diphenylamine derivative.

REFERENCES

1 DEPARTEMENTS OF THE ARMY AND THE AIR FORCE, *TM 9-1910, Military Explosives*, Government Printing Office, Washington 25, D.C., 1955, p. 240.
2 J. HANSSON AND A. ALM, *J. Chromatog.*, 9 (1962) 385.
3 W. A. SCHROEDER, E. W. MALMBERG, L. L. FONG, K. N. TRUEBLOOD, J. D. LANDERL AND E. HOERGER, *Ind. Eng. Chem.*, 41 (1949) 2818.
4 S. K. YASUDA, *J. Chromatog.*, 13 (1964) 78.

STUDIUM DER KOMPLEXVERBINDUNGEN IN LÖSUNG MITTELS PAPIERELEKTROPHORESE*

II. ELEKTROPHORETISCHE BEWEGLICHKEIT UND STABILITÄT DER EINKERNIGEN KOMPLEXE

VLADIMÍR JOKL

Lehrstuhl für Analytische Chemie, Pharmazeutische Fakultät der Komensky-Universität, Bratislava (Tschechoslowakei)

(Eingegangen den 5. Juli 1963)

Professor A. OKÁČ zum 60. Lebensjahre gewidmet

In erster Mitteilung dieser Reihe[1] wurde die Bedeutung der elektrophoretischen Beweglichkeitskurve (als einer Funktion der Konzentration des freien Liganden) beim Studium von löslichen Komplexen mit negativ geladenen Liganden dargelegt. Es wurden empirische Gleichungen für Abschätzung der Zusammensetzung der Komplexe auf Grund elektrophoretischer Beweglichkeiten in Gebieten der Plateaus der Beweglichkeitskurven abgeleitet. Die Beweglichkeit in Gebieten der sinkenden Äste der Beweglichkeitskurven ist von der Konzentration des freien Liganden abhängig. Die Lage dieser Gebiete stellt eine Information über Stabilität der entstehenden Komplexe dar.

Den Einfluss der Konzentration des Komplexbildners auf die Beweglichkeiten der Jodokomplexe vom Kadmium bei freier Elektrophorese (Tiselius) haben ALBERTY UND KING[2] untersucht. Die Autoren haben ihre Resultate auch — mit Hinsicht auf die Stabilitätskonstanten — quantitativ interpretiert. Das Verhalten der Halogenokomplexe bei der Papierelektrophorese ist von PUČAR[3] studiert worden; er hat im allgemeinen den Einfluss der Konzentration des Komplexbildners auf die Beweglichkeiten festgestellt, bzw. bestätigt. Schon vorher ist dies an Quecksilber (II)-Chlorokomplexen demonstriert worden[4]. Vom theoretischen Standpunkt ist das Problem von KÖRÖSY[5] angedeutet worden. Grundsätzlich haben sich mit der Relation der Beweglichkeit zur Konzentration des Komplexbildners und zu Dissoziationskonstanten der Komplexe WĘTZEL UND VOIGT[6] beschäftigt, und zwar im Sinne der zielbewussten Wahl bester Bedingungen für Trennung der Ionen; experimentell ist von ihnen die Problematik nicht untersucht worden.

THEORETISCHER TEIL

Ein Zentralatom M bildet mit negativ geladenen Liganden A stufenweise einkernige Komplexe MA_i:

$$M + A \rightleftarrows MA$$
$$MA + A \rightleftarrows MA_2$$
$$\vdots$$
$$MA_{n-1} + A \rightleftarrows MA_n$$

* Vorläufige Mitteilung: *Česk. Farm.*, 12 (1963) 44.

MA_n ist der höchste Komplex, welcher unter gegebenen Bedingungen entstehen kann. Ausserdem verläuft auch die Hydrolyse des Ions:

$$M(H_2O) \rightleftarrows MOH + H^+$$

Die einzelnen Komplexstufen (einschliesslich freien Zentralions) M, MA, ... MA_n, MOH weisen die elektrophoretischen Beweglichkeiten u_0, u_1, ... u_n, u_{MOH} auf. Für Komplexe mit negativ geladenen Liganden gilt immer $u_{i-1} > u_i$, wenn man die Beweglichkeit zur Kathode konventionell als positiv bezeichnet. Ist das Gleichgewicht der Komplexbildung mobil, dann kommt es bei der Migration während der Elektrophorese nicht zur Trennung auf einzelne Zonen, sondern das Gleichgewichtsgemisch wandert als einzige Zone. Ihre Beweglichkeit bezeichnen wir als aktuelle Beweglichkeit U (sonst auch "net-mobility" genannt). Diese muss im Intervall (u_0, u_n) liegen und hat einen additiven Charakter. Alle Komponenten des Gleichgewichtsgemisches tragen der aktuellen Beweglichkeit je nach ihrem Anteil im Gemisch bei:

$$U = u_0 \frac{[M]}{c_M} + u_1 \frac{[MA]}{c_M} + \cdots + u_n \frac{[MA_n]}{c_M} + u_{MOH} \frac{[MOH]}{c_M} =$$

$$= \frac{\sum_{i=0}^{n} u_i [MA_i] + u_{MOH} [MOH]}{\sum_{i=0}^{n} [MA_i] + [MOH]} \qquad (1)$$

($MA_0 = M$!).

Unter gewissen Bedingungen kann im Gleichgewichtsgemisch eine Komplexstufe weitaus überwiegen; sie bestimmt dann praktisch allein die Beweglichkeit und auf der Beweglichkeitskurve ist dies durch ein Plateau gekennzeichnet (vergl. Fig. 2 in Mitt. I[1]).

Die sinkenden Äste der Beweglichkeitskurve entstehen durch Koexistenz von mehreren nachfolgenden Komplexstufen in vergleichbaren Konzentrationen. Bei grossen Unterschieden in Stabilitäten existieren praktisch nur zwei Komplexstufen MA_{i-1} und MA_i zusammen im Gleichgewicht, und die entsprechenden Wellen an der Beweglichkeitskurve sind gut abgetrennt (z.B. die Kupfer–Glycin-Komplexe $CuGl_2$ und $CuGl_3^-$, Fig. 1 der Mitt. I). Sonst gibt es mehrere nachfolgenden Komplexstufen im Gleichgewicht, und die einzelnen Wellen superponieren sich in eine Resultante (z.B. die Komplexe $CuGl^+$ und $CuGl_2$ an der erwähnten Abbildung).

Alle ausgesprochenen Voraussetzungen können nur dann als gültig betrachtet werden, wenn der Grundelektrolyt den Komplexbildner enthält. Die Konzentration des Liganden muss räumlich und zeitlich konstant bleiben[6], also gepuffert sein. Wenn nämlich bei einer Reaktion der Komplexbildung in freier Lösung die Konzentration des freien Liganden ein Ergebnis der Einstellung des Gleichgewichtes ist, dann ist sie bei der Trägerelektrophorese im Gegenteil für die Einstellung des Gleichgewichtes in der wandernden Zone bestimmend. Ihre Konstanz gewährleistet auch die konstante Verteilung aller Komplexstufen während der Migration.

Die Forderung gepufferter Konzentration lässt sich leicht erfüllen, wenn als Liganden die Anionen einer schwachen Säure H_pA auftreten. Ihre Konzentration ist bekanntlich gleich:

$$[A] = c_{HpA} \cdot \frac{\varkappa_p}{\sum_{j=0}^{p} \varkappa_j [H^+]^{p-j}} \qquad (2)$$

$$(K_0^H = 1, \varkappa_j = K_1^H \cdot K_2^H \cdots K_j^H)$$

Die Gleichung (2), welche zur Berechnung von [A] mittels der gemessenen pH-Werte dient, zeigt zugleich, dass in einer Lösung mit gepufferter Protonenaktivität auch die Konzentration [A] gepuffert ist. Mit Ausnahme der eigenen Puffergebiete der komplexbildenden Säure muss also der Grundelektrolyt fremde Puffer enthalten, deren Komplexwirkung gegenüber dem studierten Komplexbildner allerdings vernachlässigbar sein muss.

Die aktuelle Beweglichkeit hängt von der Konzentration des freien Liganden ab. Dies geht daraus hervor, dass man in der Gleichung (1) die Gleichgewichtskonzentrationen mittels der betreffenden Komplexbildungskonstanten ausdrückt:

$$U = \frac{[M]\left(u_0 + u_1K_1[A] + u_2K_1K_2[A]^2 + \cdots + u_nK_1K_2 \cdots K_n[A]^n + U_{MOH}\frac{k_H}{[H^+]}\right)}{[M]\left(1 + K_1[A] + K_1K_2[A]^2 + \cdots + K_1K_2 \cdots K_n[A]^n + \frac{k_H}{[H^+]}\right)} =$$

$$= \frac{\sum_{i=0}^{n} u_i\beta_i[A]^i + u_{MOH}\frac{k_H}{[H^+]}}{\sum_{i=0}^{n} \beta_i[A]^i + \frac{k_H}{[H^+]}} \qquad (3)$$

($\beta_0 = 1$, $\beta_i = K_1 \cdot K_2 \ldots K_i$, k_H = erste hydrolytische Konstante des Kations M).

Die Gleichung (3) ist die allgemeine Gleichung der Beweglichkeitskurve eines einkernigen Komplexes (siehe Fig. 1). Sie zeigt, dass die Beweglichkeit nicht von der Konzentration des Zentralatoms, sondern nur von der des freien Liganden abhängig ist (unter Voraussetzung vollkommen gepufferter Konzentration [A] mit genügender Kapazität). Die Beweglichkeitskurve lässt sich zwar auch als Funktion des pH-Wertes des Grundelektrolyten konstruieren, aber für die Abschätzung von Stabilitäten ist die Abhängigkeit von [A] geeigneter. Es ist weiter ersichtlich, dass im Falle

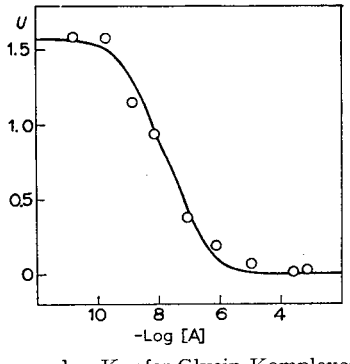


Fig. 1. Die Beweglichkeitskurve des Kupfer-Glycin-Komplexes. O—O Experimentalpunkte: $c_{Cu} = c_{Gl} = 5 \cdot 10^{-2}$; T = 20°; μ = 0.1. ——— Kurve berechnet nach Gleichung (3): log K_1 = 8.62, log K_2 = 6.97 (Lit. 7); u_0 = 1.57, u_1 = 0.81.

eines nicht zu grossen Hydrolysekonstanten und nicht zu schwacher Komplexe der Einfluss der Hydrolyse völlig vernachlässigbar ist (z.B. mittelstabile Komplexe der zweiwertigen Übergangsmetalle).

Die Ermittlung der Komplexbildungskonstanten durch Auswerten einiger Experimentalpunkte gibt in Bezug auf die verminderte Genauigkeit der elektrophoretischen Methode inkonsistente Ergebnisse. Viel besser ist eine graphische Auswertung der Beweglichkeitskurve, die selbst schon eine gewisse Ausgleichung darstellt. Man bestimmt den Punkt $U_{1/2}$ auf der Beweglichkeitskurve, für welchen die Bedingung

$$K_i \cdot [A] = 1 \text{ (oder } \log K_i = -\log [A]) \tag{4}$$

gültig ist:

$$U_{1/2} = \frac{u_0 + \cdots + u_{i-1}\beta_{i-1}[A]^{i-1} + u_i\beta_i[A]^i + \cdots}{1 + \cdots + \beta_{i-1}[A]^{i-1} + \beta_i[A]^i + \cdots} \tag{3a}$$

In bezug auf Gleichung (4) gilt es:

$$\beta_i \cdot [A]^i = \beta_{i-1} \cdot [A]^{i-1}$$

Bei grossen Unterschieden der Komplexbildungskonstanten ist ausserdem

$$1 \ll K_1[A] \ll \cdots \ll \beta_i[A]^i \gg \beta_{i+1}[A]^{i+1} \gg \cdots$$

Die Gleichung (3a) geht dann in die vereinfachte Form über:

$$U_{1/2} = \frac{u_{i-1} + u_i}{2} \tag{5}$$

Durch Ablesen der zugehörigen Werte von [A] bekommt man laut Gleichung (4) die annähernden Werte von K_i.

Bei mehrstufiger Komplexbildung sind die oben angeführten Beziehungen nur dann genau gültig, wenn die einzelnen Wellen der Beweglichkeitskurve gut ausgebildet und abgetrennt sind (d.h. bei grossen Unterschieden der Konstanten). Sonst existieren mehr als zwei Komplexstufen nebeneinander, und die ermittelten Konstanten sind als approximative Werte anzusehen, die mit geringeren oder grösseren Fehlern belastet sind. Berechnung genauerer Konstanten mittels fortschreitender Approximationen ist wegen der kleinen Genauigkeit der elektrophoretischen Methode selbst nicht zweckmässig.

EXPERIMENTELLES

Die Arbeitsmethodik, Versuchsanordnung und Auswertung der Ergebnisse wurden in der vorangegangenen Mitteilung[1] beschrieben. Alle Beweglichkeiten sind als relative Werte auf $(C_2H_5)_4N^+$ bezogen ($u = 1.00$), unter Berücksichtigung der Elektroosmose.

Die für Konstruktion von Beweglichkeitskurven nötigen Werte von [A] wurden nach der Gleichung (2) berechnet.

Versuchsmaterial

Die Grundelektrolyte enthielten (wenn nicht anders angegeben) die komplexbildende Säure in 0.05 *M* Konzentration. Ausserhalb der eigenen Puffergebiete der Säuren

wurden Chloracetat-, Formiat-, Acetat-, Veronal- oder Borat-puffer in 0.05 M Konzentration zugegeben. Die Ionenstärke wurde je nach Gebrauch auf den Wert $\mu = 0.1$ mit KNO_3 aufgestellt. Der pH-Wert der bereiteten Lösungen wurde mit dem Kompensations-pH-meter PHK 1, Mikrotechna-Prag (hochohmige Glaselektrode) gemessen.

Die Konzentration der aufzubringenden Komplexe war ebenfalls 0.05 M; die Lösungen wurden vor dem Aufbringen annähernd auf den pH-Wert des betreffenden Grundelektrolyten aufgestellt (aber nur wenn keine Fällung entstand).

ERGEBNISSE UND DISKUSSION

Die Fig. 2 stellt einige typischen Beweglichkeitskurven von Komplexen dar, an welchen die Grundrisse der qualitativen Interpretation der Kurven gezeigt werden sollen. Die Kurve des Kupfer-glycinats (1) zeigt die Bildung eines elektroneutralen Kom-

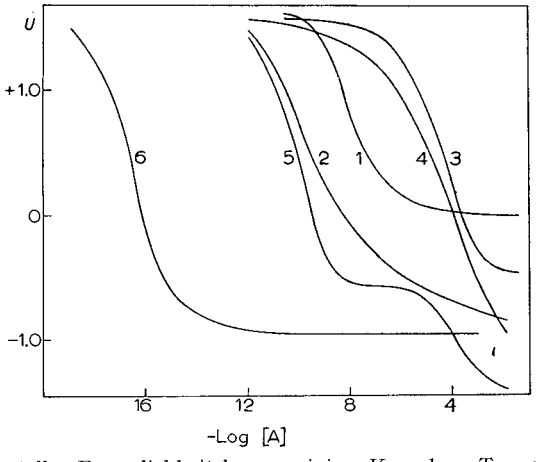


Fig. 2. Die experimentellen Beweglichkeitskurven einiger Komplexe. T = 20°; $c_M = c_A = 5 \cdot 10^{-2}$; $\mu = 0.1$ mit Ausnahme eines Teils der Kurven 5 und 6. (1) Kupfer–Glycin; (2) Kupfer–Glutaminsäure; (3) Zink–Glycin; (4) Zink–Glutaminsäure; (5) Kobalt (II)–Nitrilotriessigsäure; (6) Kobalt (II)–Äthylendiamino-tetraessigsäure ($c_A = 10^{-3}$).

plexes; in den untersuchten Versuchsbedingungen entsteht also der Komplex $CuGl_2$. Auch mit der Glutaminsäure (2) bildet sich höchstens Kupferkomplex 1:2, welcher nach der empirischen Gleichung[1]:

$$\frac{u}{z} = 14.7 \frac{1}{\sqrt{\bar{m}}} - 0.29 \qquad (6)$$

die Beweglichkeit —0.98 besitzen soll. Dagegen die Zinkkomplexe von Glycin (3) und Glutaminsäure (4) weisen wesentlich negativere Beweglichkeiten auf, die offenbar auf die Komplexe 1:3 hinweisen. Beweglichkeit des Zink–Glycin-komplexes nach Gleichung (6) ist —0.58 (exptl. —0.46); die Beweglichkeit des höchsten Zink–Glutamatokomplexes lässt sich aus der Kurve nicht bestimmen, sie sollte nach Gleichung (6) —1.48 betragen. Die geringere Stabilität der Zinkkomplexe gegenüber den Kupfer-

komplexen ist schon an der Verschiebung der Kurven in Richtung höherer Konzentrationen von [A] qualitativ ersichtlich.

Die Beweglichkeitskurve des Kobalt (II)-komplexes der Nitrilotriessigsäure (5) weist zwei gut abgetrennte Wellen auf. Die erste gehört dem Komplex $[CoX(H_2O)_2]^-$ (Beweglichkeit ber. —0.58, gef. —0.56), die zweite dem höheren Komplex $[CoX_2]^{4-}$ (Beweglichkeit ber. —1.64). Mit der Äthylendiamino-tetraessigsäure entsteht lediglich der Komplex $[CoY]^{2-}$ mit der Beweglichkeit —0.96 (ber. —1.0).

Die Stabilität einer Reihe von Komplexen verschiedener Aminosäuren mit zweiwertigen Übergangsmetallen wurde mit Hilfe der Gleichung (5) untersucht. Werte

TABELLE I

STABILITÄTSKONSTANTEN EINIGER KOMPLEXE

(T = 20°, μ = 0.1)

Metall	*Komplexbildner*	*log* K_1		*log* K_2		*log* K_3	
		Exptl.	*Literatur*	*Exptl.*	*Literatur*	*Exptl.*	*Literatur*
Cu	Glycin	8.6	8.62	7.2	6.97	0.15	0.33
Co(II)		5.5	5.23	3.5	4.02	2.3	—
Ni		6.4	6.18	4.4	4.98	3.0	—
Cd		6.0	—	3.9	8.1*	2.6	—
Mn		3.9	3.84	1.7	1.66	—	—
Zn		5.9	5.52	4.2	4.44	3.1	2.6
Cu	Alanin	8.5	8.51	6.7	6.86	—	—
Co(II)		5.0	4.82	3.2	3.66	2.4	—
Ni		6.0	5.96	4.3	4.70	2.9	—
Cd		5.9	—	3.5	—	2.4	—
Mn		3.4	3.02	1.9	3.03(?)	—	—
Zn		5.7	5.21	3.9	4.33	2.3	—
Cu	Leucin	8.6	—	7.0	14.34*	—	—
Co(II)		5.2	—	3.2	8.25*	2.3	—
Ni		6.3	—	4.0	—	2.5	—
Cd		5.8	—	3.6	—	2.4	—
Mn		3.9	—	1.8	5.45*	—	—
Zn		5.8	—	4.2	8.93*	3.3	—
Cu	Methionin	8.1	—	6.7	14.7*	—	—
Co(II)		4.5	—	3.1	7.9*	1.9	—
Ni		5.7	—	3.7	—	2.3	—
Cd		5.4	—	3.3	—	2.1	—
Mn		3.2	—	(1.5)	—	—	—
Zn		4.9	—	3.6	—	(3.2)	—
Cu	Glutaminsäure	10.1	—	6.3	—	—	—
Co(II)		4.9	—	3.1	—	—	—
Ni		5.8	—	3.4	—	—	—
Cd		5.3	4.39	2.9	—	—	—
Mn		3.4	—	—	—	—	—
Zn		5.6	—	3.2	—	—	—
Cu	Nitrilotriessigsäure	11.5	12.7	3.3	—	—	—
Co(II)		10.0	10.6	3.4	3.9	—	—
Cd		10.0	9.8	4.6	5.7	—	—
Mn		8.6	7.4	3.0	3.7	—	—

* $\log K_1 \cdot K_2$.

der Beweglichkeiten, die sich nicht experimentell erfassen lassen, wurden nach der Gleichung (6) berechnet. In der Tabelle I sind die gefundenen annähernden Komplexbildungskonstanten mit den potentiometrisch (pH) bestimmten[7] verglichen, um die Tragweite, Möglichkeiten und Genauigkeit der elektrophoretischen Methode kritisch beurteilen zu können. Natürlich handelt es sich um Konzentrationskonstanten, weil sie nicht auf Aktivitäten, sondern auf Konzentrationen der beteiligten Stoffe bezogen sind. Einige der angegebenen Konstanten wurden bisher mit anderen Methoden nicht bestimmt.

Die Versuchsbedingungen bei der Papierelektrophorese können nicht genügend konstant gehalten werden und ausserdem ist die Bestimmung der Beweglichkeiten nicht allzu genau (auf 3-5% reproduzierbar). Deswegen muss man die ermittelten Konstanten vor allem als Orientierungswerte ansehen. Am besten lassen sich Komplexe von mittlerer Stabilität untersuchen (log K etwa 3–12). Für schwache Komplexe sind hohe Konzentrationen vom Komplexbildner und Puffern erforderlich, was auf technische Hindernisse stösst (vor allem auf ein zu grosses Leitvermögen der Lösungen). Bei sehr stabilen Komplexen ist manchmal die Bedingung eines mobilen Reaktionsgleichgewichtes nicht mehr erfüllt; ausserdem gibt es Schwierigkeiten mit der Pufferung in extrem niedrigen Konzentrationsbereichen.

Die Methode besitzt aber einige Vorteile. Als ein Trennverfahren erlaubt sie auch bei Anwesenheit fremder Stoffe zu arbeiten (soweit sie nicht in den Migrationsvorgang direkt eingreifen) und der Aufwand an untersuchten Metallsalzen ist äusserst gering. Im Vergleich mit anderen physikalisch-chemischen Methoden für das Studium der Komplexe in Lösung gibt sie eine direkte Auskunft über die Art, Zusammensetzung oder Ladung der entstehenden Körper. Sie stellt somit einen guten Ausgangspunkt für Anwendung sonstiger genauerer Untersuchungsmethoden.

DANK

Der Autor fühlt sich Herrn Dr. D. DYRSSEN, Stockholm, mit dem besten Dank für eine wertvolle Bemerkung zu dieser Arbeit verpflichtet.

ZUSAMMENFASSUNG

Es wurde die allgemeine Gleichung der Beweglichkeitskurve für lösliche einkernige Metallkomplexe mit Anionen schwacher Säuren abgeleitet und ihre Geltung an einer Reihe von Komplexen der zweiwertigen Metallionen mit einigen Aminosäuren bestätigt. Eine graphische Methode für Bestimmung der annähernden Komplexbildungskonstanten aus der elektrophoretischen Beweglichkeitskurve wurde vorgeschlagen, und die gefundenen Konstanten mit den potentiometrisch bestimmten verglichen. Die Beschränkungen und Vorteile der Methode wurden besprochen.

SUMMARY

The general equation for the electrophoretic mobility curve of soluble mononuclear complexes of metals with anions of weak acids is derived. The validity of this equation is confirmed in the case of complexes of some bivalent metal ions with several amino acids. A graphic method for the determination of the approximate stability constants

from the electrophoretic mobility curves is proposed, and the electrophoretic values are compared with the potentiometric values. The limitations and advantages of the method are discussed.

LITERATUR

[1] V. JOKL, *J. Chromatog.*, 13 (1964) 451.
[2] R. A. ALBERTY UND E. L. KING, *J. Am. Chem. Soc.*, 73 (1951) 517.
[3] Z. PUČAR, *Anal. Chim. Acta*, 17 (1957) 476, 485; 18 (1958) 290.
[4] K. A. KRAUS UND G. W. SMITH, *J. Am. Chem. Soc.*, 72 (1950) 4329.
[5] F. KÖRÖSY, *Magy. Kém. Folyóirat*, 60 (1954) 101.
[6] K. WETZEL UND G. VOIGT, *Z. Phys. Chem.*, 216 (1961) 7.
[7] A. E. MARTELL UND M. CALVIN, *Chemistry of the Metal Chelate Compounds*, Prentice-Hall, New York, 1956.

SEPARATION OF RARE EARTHS ON ANION EXCHANGE RESINS

IV. INFLUENCE OF TEMPERATURE ON ANION EXCHANGE BEHAVIOUR OF THE RARE EARTH ETHYLENEDIAMINETETRAACETATES*

RAJMUND DYBCZYŃSKI

Analytical Chemistry Division of the Institute of Nuclear Research, Warsaw (Poland)

(Received July 11th, 1963)

Raising the temperature in order to improve the quality of separation has often been applied in ion exchange chromatography. The rise of temperature causes the diffusion coefficients to increase in the solution as well as in the resin phase and accelerates the stabilisation of local equilibrium, and hence the height (H) equivalent of the theoretical plate diminishes. The smaller H is, the narrower are the elution curves of the separate components of the mixture, and the better the separation.

Most of the previous work in which increased temperatures were applied, *viz.* in chromatography of rare earths and actinides on cation exchange resins with the use of organic acids as eluants, concerned systems in which separation is brought about by differences in the stability constants of complexes formed by the metal ions with eluant ions and not by differences in the affinity of ions for the ion exchange resin.

In such systems the change of temperature only effects the absolute values of the distribution coefficients while the ratio of the distribution coefficients, the separation factor (α) remains practically unchanged[1,2]. In this case, the positive influence of the rise of temperature is exclusively concerned with decreasing the height equivalent of the theoretical plate.

In systems in which the different rates of migration of the components of a mixture down the column are due to the differences in the individual affinity of the ions for the ion exchange resin, the change of temperature has a double effect:

(a) the height equivalent of the theoretical plate (H) diminishes with rise of temperature as usual; and

(b) the values of the distribution coefficients of the individual ions undergo changes in accordance with the values and signs of the enthalpies of the ion exchange reaction, and hence the separation factor (α) changes as well.

This second aspect of the influence of temperature has not yet been explained in the literature.

Up to now, it has been tacitly assumed that the exchange reactions' enthalpies are so small that changes of temperature cannot be of any great consequence for the course of separation.

However, when dealing with ions with very similar distribution coefficients, even small changes of the separation factor can be of great practical importance. The

*For Part III, see ref.[11].

values of the enthalpies of ion exchange reactions are of the order of several kcal[3–7]. This means that in the universally accessible range of temperature of 0–100°C the value of the distribution coefficient of a given ion can increase (or decrease) several times. At the same time the separation factor of two ions can increase several times especially when the enthalpy signs of the two ion exchange reactions are opposite. Rise of temperature still has one favourable effect, namely, it decreases the viscosity of solutions and thus diminishes the hydraulic resistance and permits the use of greater flow rates. In previous papers[8–11], a new method of separation of rare earths on anion exchange resins was discussed which took advantage of differences in the affinity of rare earth complexes with ethylenediaminetetraacetic acid (H_4Y) to ion exchange resin.

The preliminary experiments have already proved that a change of temperature has a great influence on the separation factor for particular rare earths, and in some cases the sequence of elution is even reversed. The detailed results of the investigation of the thermodynamics of anion exchange of the rare earth ethylenediaminetetraacetates have been given in another paper[6].

The present publication deals with analytical aspects of the separation of rare earths in the system: Amberlite IRA-400 [H_2Y^{2-}]–Na_2H_2Y aq.

EXPERIMENTAL

The ion exchange resin

The strongly basic anion exchange resin Amberlite IRA-400 [Cl^-] was ground in a ball-mill, sieved and the fraction of particle size < 0.07 mm (200 mesh) was collected. This resin was further separated into fractions according to their settling time in water. In this way a fraction was collected in which the diameter of more than 95 % of the particles was within the range of 10–35 μ, as shown by microscopic examination.

This ion exchange resin was successively treated in a glass column with a large excess of 1 *N* NaOH and 1 *N* HCl. After washing with water, the resin was transformed into ethylenediaminetetraacetate [H_2Y^{2-}] form by passing 0.2 *M* disodium-ethylenediaminetetraacetate solution (Na_2H_2Y) down the column until no chlorides could be detected in the effluent. Then ion exchanger was washed with deionized water and air dried.

In order to determine the exchange capacity a known weight of the resin was put in the column; the [H_2Y^{2-}] ions were eluted with 1 *M* NaCl solution and determined by titration with a standard solution of magnesium chloride in presence of Eriochrome Black T. The quantity of water in the anion exchange resin was determined by drying at 105°C constant weight.

The exchange capacity was found to be $Z_s = 2.38$ mval/g of the dry ion exchange resin [H_2Y^{2-}].

The bed density has been previously determined and amounted to $d_z = 0.326$ g of the dry ion exchange resin [H_2Y^{2-}]/ml of the bed.

Reagents

Disodium-ethylenediaminetetraacetate was prepared from commercial $Na_2H_2Y \cdot 2H_2O$ by purifying it by the method of BLAEDEL AND KNIGHT[12]. Solutions of the required concentration were prepared by dissolving the appropriate quantity of pure $Na_2H_2Y \cdot 2H_2O$ in deionized water. The exact concentration of the solution

was determined by titrating it with standard solution of magnesium chloride in presence of Eriochrome Black T. Since in most cases very dilute Na_2H_2Y solutions were used, the density values of pure water given in the literature were applied in order to convert molarity into molarity. The pH values of the Na_2H_2Y aq. solutions were within the range of 4.55–4.70.

Radioactive tracers

The following radioactive tracers were used: ^{134}Cs (half life = 2.2 years); ^{140}La (half life = 40 h); ^{144}Ce (half life = 285 days); ^{142}Pr (half life = 19.2 h); ^{147}Pm (half life = 2.6 years); $^{152,154}Eu$ (half life = 12.2 years); ^{159}Gd (half life = 18.0 h); ^{160}Tb (half life = 73.5 days); ^{166}Ho (half life = 27.3 h); ^{170}Tm (half life = 127 days); ^{177}Lu (half life = 7.0 days); ^{46}Sc (half life = 85 days); ^{90}Y (half life = 65 h).

The radioactive tracers ^{144}Ce, ^{147}Pm and $^{152,154}Eu$ were supplied by Sojuzchim-Export, USSR.

The others were prepared by irradiating the appropriate spectral pure grade of oxides or chlorides (Johnson Matthey "Specpure") with neutrons in the Polish reactor EWA.

Apparatus

The apparatus used here was similar in principle to that described in a previous paper[19]. The only substantial difference consisted in using the jacketed columns. The temperature was maintained constant within the limits of $\pm$ 0.4°C by passing water from a Höppler ultrathermostat through the jacket. The internal diameter of the actual column was 2.1–2.7 mm. The height of the ion exchange resin bed was altered according to actual needs.

Procedure

The column filled with Amberlite IRA-400 [H_2Y^{2-}] was rinsed with several millilitres of the eluant (the Na_2H_2Y solution of the known molarity). When the desired temperature had been obtained and the column brought to thermal equilibrium the level of the liquid in the column was adjusted to the upper level of the resin bed.

The solution of the radioactive tracers of rare earths (in HCl) together with a small quantity of ^{134}Cs was evaporated to dryness in a glass crucible, then the theoretical quantity of Na_2H_2Y solution necessary to form complexes with all the rare earths was added to the crucible which was again evaporated to dryness. The residue was dissolved in 75 μl of the eluant, and 25 μl of this solution were introduced into the column. The total rare earth content in the "load" did not exceed 0.3 % of the exchange capacity of the ion exchange resin in the column. After the solution has passed into the resin bed, the walls of the column were rinsed with two 10 μl portions of the eluant. A burette was connected to the column and the flow rate adjusted to about 1 ml/cm^2/min. The volume of a drop was determined by noting burette readings corresponding to a known number of drops of eluant. The drops previously dried on paper were cut out with the aid of a special cutter, placed in standard plexiglass holders, and their activity was measured under identical geometrical conditions with the aid of an end-window Geiger-Müller counter. The elements corresponding to particular maxima on the elution curve were identified by determining the half life or the maxi-

mum energy of radiation, or by comparing them with the elution curves obtained for single elements under identical conditions.

The fractional free volume of the ion exchange resin bed, $i = v/v_b$ (the ratio of the free volume to the bed volume), was determined from the changes of the peak position of the ^{134}Cs ion, for which the distribution coefficient, λ, in the given system is equal to 0:

$$i = \frac{U_{max2} - U_{max1}}{v_{b2} - v_{b1}},$$

where U_{max_2} and U_{max_1} are the effluent volumes corresponding to the maxima of the caesium peak for the volumes of the ion exchange resin bed v_{b_2} and v_{b_1} respectively.

The fractional free volume thus determined amounts to $i = 0.42$.

The method of determining the free volume (formula (2)[10]) previously applied gave results which were distinctly too high.

Although strongly basic anion exchange resins in the "salt" form are more resistant to the effects of higher temperatures than they are in the hydroxide form[13], the ion exchange resin in the column was changed after each experiment where the temperature exceeded 45°C in order to avoid any complications caused by possible degradation of the functional groups[14].

RESULTS AND DISCUSSION

Formulation of fundamental relations

Previous work[6,9–11] has shown that in Na_2H_2Y aq. solution (pH = 4.55 — 4.70) practically 100 % of all the rare earths appear in the form of their respective monovalent negative $[LnY(H_2O)]^-$ complexes. Under these conditions nearly 100 % of ethylenediaminetetraacetic acid also appears in the form of the H_2Y^{2-} anion. The ion exchange reaction (with respect to one equivalent) can be expressed as follows:

$$\tfrac{1}{2}\,R_2H_2Y + LnY^- = RLnY + \tfrac{1}{2}\,H_2Y^{2-} \qquad (1)$$

where R is the cationic part of the anion exchange resin.

Without taking into consideration the activity coefficients, the equilibrium constant of the exchange reaction, or the so called "selectivity coefficient", is given by the equation:

$$k = \frac{N_{RLnY} \cdot m^{\frac{1}{2}}_{H_2Y^{2-}}}{N^{\frac{1}{2}}_{R_2H_2Y} \cdot m_{LnY^-}} \qquad (2)$$

where N is the mole fraction of the ion in the ion exchange resin and m is molality.

When the separated ions (in this case the LnY^- ions) are present in trace amounts *i.e.* $N_{R_2H_2Y} \cong 1$, the selectivity coefficient k is related to the weight distribution coefficient, λ (amount per 1 g of the dry ion exchange resin $[H_2Y^{2-}]$/amount per 1 ml of the solution), by the following expression[6]:

$$k^{LnY^-}_{H_2Y^{2-}} = \frac{\lambda_{LnY^-} \cdot m^{\frac{1}{2}}_{H_2Y^{2-}} \cdot d}{C_r} \qquad (3)$$

where d = the density of the eluant solution, and

C_r = the concentration of the resin phase in the units of mmol./g of the dry ion exchange resin $[H_2Y^{2-}]$; $(C_r = {}^1/_2 Z_s)$.

The distribution coefficient can easily be determined from the elution curve[10] by the formula:

$$\lambda = \frac{U_{max} - (U_o + V)}{m_j} \tag{4}$$

where U_{max} = volume of the eluant at which the concentration of the given ion reaches its maximum;

U_o = dead volume of the column;

V = free volume of the ion exchange resin bed; and

m_j = mass of the dry ion exchange resin in the column (in grams).

Since the Cs^+ ions do not form any complexes with H_4Y and are not retained by the anion exchange resin in the ethylenediaminetraacetate form[10,15];

$$\lambda_{LnY^-} = \frac{U_{max(LnY^-)} - U_{max(Cs^+)}}{m_j} \tag{5}$$

The k values, determined experimentally for several temperatures, can be use to compute the respective changes of free energy (ΔG), enthalpy (ΔH) and entropy (ΔS). Strictly speaking the thermodynamic functions so obtained are partial molal values for $X_{LnY^-} \cong 0$ (X_{LnY^-} is the equivalent fraction of the LnY^- ion in the ion exchange resin).

The method of computation and the exact values of ΔH^*, ΔG^* and ΔS^* for temperatures within the range 2–92°C have been given in another paper[6]. As a of interest, the values of these functions for the temperature 298°K (25°C) is shown in Table I.

TABLE I

THE THERMODYNAMIC FUNCTIONS FOR THE ION EXCHANGE REACTION:

$${}^1/_2\, R_2H_2Y + LnY^- \rightleftharpoons RLnY + {}^1/_2\, H_2Y^{2-}$$

Ln	*Atomic number Z*	ΔG^*_{298}°K *kcal/equiv.*	ΔH^*_{298}°K *kcal/equiv.*	ΔS^*_{298}°K *cal/equiv.*	ΔC^*_p *cal/equiv.* °K
Sc	21	—0.270	—0.135	0.45	14.1
Y	39	—0.561	—2.079	— 5.09	36.2
La	57	—1.017	2.251	10.97	7.8
Ce	58	—1.526	1.721	10.89	—0.7
Pr	59	—1.716	2.318	13.54	—33.6
Nd*	60	—1.896	2.172	13.65	—58.9
Pm	61	—2.027	1.962	13.39	—82.1
Sm*	62	—2.087	1.059	10.56	—81.8
Eu	63	—2.118	—0.225	6.35	—70.6
Gd	64	—1.961	—0.467	5.01	—73.5
Tb	65	—1.678	—1.177	1.68	—55.6
Dy*	66	—1.289	—2.016	— 2.44	—20.2
Ho	67	—0.915	—2.359	— 4.85	2.7
Er*	68	—0.526	—2.153	— 5.46	21.5
Tm	69	—0.189	—2.073	— 6.32	43.6
Yb*	70	—0.132	—1.857	— 5.79	41.8
Lu	71	—0.070	—1.998	— 6.47	42.4

* The thermodynamic functions for these elements were calculated by the method previously described[6] from the values of selectivity coefficients estimated by interpolation in the $\log k$–Z curves (Fig. 3).

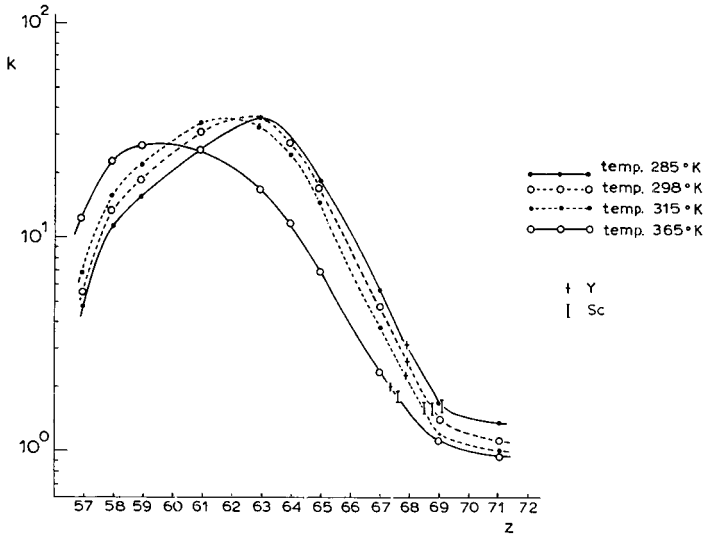


Fig. 1. Selectivity coefficients for the ion exchange reaction: $^1/_2\ R_2H_2Y + LnY^- \rightleftharpoons RLnY + {}^1/_2\ H_2Y^{2-}$, as a function of temperature. Points = experimental values; solid lines are calculated by the least squares' method[6].

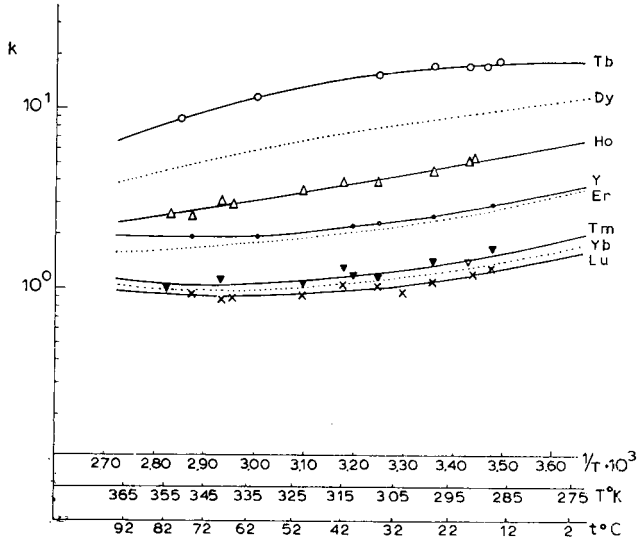


Fig. 2. Selectivity coefficients for the ion exchange reaction: $^1/_2\ R_2H_2Y + LnY^- \rightleftharpoons RLnY + {}^1/_2\ H_2Y^{2-}$, as a function of temperature. Points = experimental values; solid lines are calculated by the least squares' method[6].

The log k values for separate exchange reactions as the function of $1/T$ are given in Figs. 1 and 2.

Fig. 3 shows the plot of log$k$ *vs.* the atomic number of the lanthanide Z.

As can be seen from Fig. 3 the log$k-Z$ curves consist as it were of two slopes within which log$k$ increases or decreases monotonically with increase of Z.

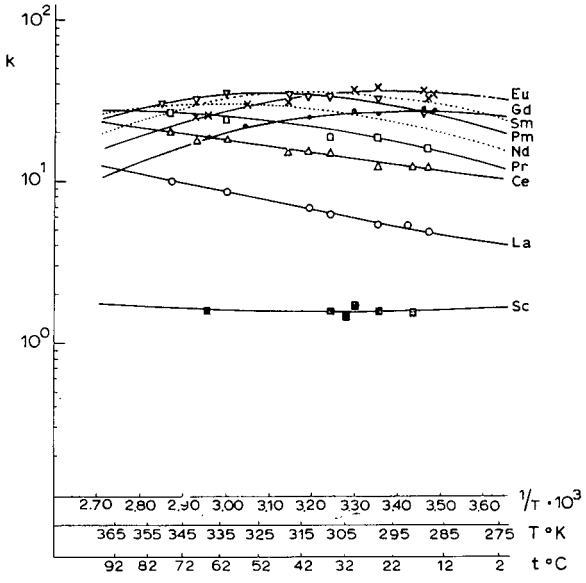


Fig. 3. Selectivity coefficients of the lanthanides in the system: Amberlite IRA-400 [$H_2Y_2^-$]–Na_2H^2Y aq., as a function of atomic number.

Owing to this, the values of log$k$ for the elements which were not investigated (Nd,Sm,Dy,Er,Yb) could be determined with reasonable accuracy. These results are shown in Figs. 1 and 2 by broken lines.

Separation factors

The separation factor α (the ratio of the distribution coefficients of the components being separated) is a value of fundamental importance in ion exchange chromatography. With an increase in the separation factor a less efficient column *i.e.* the column of a smaller number of theoretical plates, is sufficient to carry out the separation required[16–17].

From equation (3) the following ratio is obtained:

$$\alpha_{Ln(2)}^{Ln(1)} = \frac{\lambda\, Ln(1)\, Y^-}{\lambda\, Ln(2)\, Y^-} = \frac{k_{H_2Y^{2-}}^{Ln(1)Y^-}}{k_{H_2Y^{2-}}^{Ln(2)Y^-}} \qquad (6)$$

From the data shown in Figs. 1 and 2 the separation factor can be computed for a deliberately chosen pair of rare earths at a given temperature. The values of the separation factors of the rare earths (with respect to europium) are given in Table II,

TABLE II

SEPARATION FACTORS OF THE RARE EARTHS IN THE SYSTEM AMBERLITE IRA-400 [H_2Y^{2-}]–Na_2H_2Y aq. AT DIFFERENT TEMPERATURES

Element	*Atomic number Z*	α_{Eu}^{Ln}						α_{Z+1}^{Z}					
		2°	22°	42°	62°	82°	92°	2°	22°	42°	62°	82°	92°
La	57	0.12_6	0.15_5	0.20_9	0.32_9	0.54_9	0.72_6	0.39_9	0.43_4	0.44_8	0.48_3	0.52_2	0.54_4
Ce	58	0.31_6	0.35_6	0.46_7	0.68_1	1.05	1.34	0.83_5	0.73_6	0.70_7	0.73_1	0.78_8	0.83_1
Pr	59	0.37_8	0.48_4	0.66_1	0.93_2	1.34	1.61	0.75_6	0.73_9	0.76_9	0.82_5	0.82_4	0.99_4
Nd	60	0.59_0	0.65_5	0.86_0	1.13	1.45	1.62	0.80_9	0.80_1	0.82_7	0.89_7	1.01	1.07
Pm	61	0.61_8	0.81_8	1.04	1.26	1.43	1.51	0.78_1	0.88_1	0.95_4	1.05	1.16	1.20
Sm	62	0.79_1	0.92_9	1.07	1.20	1.23	1.26	0.79_1	0.92_9	1.09	1.20	1.23	1.26
Eu	63	1.00	1.00	1.00	1.00	1.00	1.00	1.27	1.30	1.34	1.38	1.42	1.44
Gd	64	0.78_9	0.76_7	0.74_8	0.72_5	0.70_4	0.69_2	1.41	1.60	1.69	1.73	1.71	1.68
Tb	65	0.55_9	0.47_9	0.44_2	0.42_0	0.41_1	0.41_0	1.57	1.89	1.96	1.93	1.80	1.72
Dy	66	0.35_6	0.25_4	0.22_5	0.21_8	0.22_8	0.23_8	1.77	1.87	1.96	1.90	1.80	1.71
Ho	67	0.20_1	0.13_6	0.11_5	0.11_5	0.12_7	0.13_9	1.89	1.94	1.80	1.69	1.54	1.48
Er	68	0.10_7	0.07_{00}	0.06_{38}	0.06_{82}	0.08_{26}	0.09_{42}	1.76	1.75	1.78	1.65	1.51	1.41
Tm	69	0.06_{09}	0.04_{00}	0.03_{59}	0.04_{12}	0.05_{48}	0.06_{66}	1.17	1.10	1.11	1.10	1.06	1.08
Yb	70	0.05_{21}	0.0363	0.03_{22}	0.03_{73}	0.05_{15}	0.06_{18}	1.07	1.11	1.08	1.07	1.11	1.09
Lu	71	0.04_{86}	0.03_{26}	0.02_{99}	0.03_{47}	0.04_{66}	0.05_{69}						
Sc	21	0.05_{05}	0.04_{42}	0.04_{80}	0.06_{10}	0.08_{54}	0.10_4						
Y	39	0.11_3	0.07_{49}	0.06_{70}	0.07_{53}	0.09_{73}	0.11_6						

together with the separation factors of adjacent elements computed for 20-degree intervals within the range of temperature of 2–92 °C.

It can be seen in Table II that the separation factors of rare earths in the Amberlite IRA-400 [H_2Y^{2-}]–Na_2H_2Y aq. system depend to a high degree on the temperature. This is an undisputed consequence of the fact that the value and sign of enthalpies:

$$\Delta H^* = -2.303\, R \frac{\mathrm{d}\log k}{\mathrm{d}\left(\frac{1}{T}\right)},$$

of the exchange reaction expressed by equation (1) are different for particular rare earths (see Figs. 1 and 2, and Table I); also ΔH^* is a function of temperature[6]. ($\Delta H^* = \Delta H_0^* + \Delta C_p^*.T$) and the heat capacities ΔC_p^* are different for different rare earths (*cf.* Table I). The separation factors relative to europium, which are < 1 for all the rare earths at lower temperatures, can have the values > 1 as the temperature rises (in case of the lanthanides of the cerium group). For some elements the sequence of the elution has been shown to be reversed. The maximum on the log$k$–Z curve, which for low temperatures occurs in the position of europium ($Z = 63$), moves along the axis as the temperature rises to smaller values of Z, and at 365 °K it occurs in the region of the Pr–Nd pair ($Z = 59$–60). In the light of these findings, it becomes clear that the selectivity coefficient values given previously[10,11] must be of dubious value since the room temperature at which those measurements were made was not precisely defined and it could change by as much as 10–15 degrees according to the season.

The fact that the course of the log$k$–$1/T$ curves is quite different for particular rare earths is very interesting when one considers the well known similarity of these elements and the fact that they form complexes of identical stoichiometric composition with H_4Y. The discussion on the values of thermodynamic functions[6] seems to have proved the hypothesis[9,10] put forward previously as to the structural changes of the LnY^- complex with respect to the changes of the central ion radius. The properties of the complex ion are likely to depend not only on the dimensions on the central ion but also on its electronic structure, and hence on the presence or absence of the 4f electrons. From the analytical point of view it is important that by changing the temperature it is possible to influence the separation factors of particular rare earths and in some cases to reverse the sequence of elution.

The number of theoretical plates and the resolution

The number of theoretical plates N in the column can be computed with the aid of the theory of MAYER AND TOMPKINS[18] from the formula derived previously[19]:

$$N = 8\left(\frac{C}{C+1}\right)\frac{(U_{max} - U_0)^2}{W^2} \qquad (7)$$

where apart from the symbols already explained,

C = distribution ratio, *i.e.* the ratio of the concentration of the substance in the ion exchange resin to the concentration in the solution on one theoretical plate;

W = width of the peak for the $M = M_{max}/e = 0.368 \cdot M_{max}$ ordinate.

The expression $C/(C + 1)$ for $C > 10$, *i.e.* for the majority of cases given in this paper, is close to 1 and formula (7) takes the form:

$$N = 8 \frac{(U_{max} - U_0)^2}{W^2} \tag{8}$$

Formula (8) is identical with the formula computed by GLUECKAUF[16] when taking advantage of the "continuous flow" model instead of the "discontinuous flow" model applied by MAYER AND TOMPKINS. According to the theory, the elution curves of particular components (peaks) have the form of the normal Gaussian distribution curves. As a result of the properties of normal diffusion:

$$w = \sqrt{2} \cdot 2\sigma \tag{9}$$

where σ is the dispersion (standard deviation) of the peak.

Substituting the above equation in the equation (8), we have:

$$N = \frac{(U_{max} - U_0)^2}{\sigma^2} \tag{10}$$

The height equivalent of the theoretical plate H can now easily be obtained from the formula:

$$H = \frac{L}{N} = \frac{L\,\sigma^2}{(U_{max} - U_0)^2} \tag{11}$$

where L is the length of the column.

The value of H can also be derived from the theoretical formula[20,21]:

$$H = 1.64\,r + \frac{\lambda'}{(\lambda' + i)^2} \cdot \frac{0.142\,r^2\,u}{D_s} + \frac{(\lambda')^2}{(\lambda' + i)^2} \cdot \frac{0.266\,r\,u}{(1 - i)\,D_L\,(1 + 70\,r\,u)} \tag{12}$$

where r = the radius of the ion exchange resin particle,
λ' = the bed distribution coefficient ($\lambda' = \lambda - dz$),
i = the fractional free volume of the bed,
D_s, D_L = the diffusion coefficients in the ion exchange resin and in the solution respectively, and
u = the linear flow rate of the eluant solution.

It can be seen from (12) that in the case when the deciding factor for the reaction time is the diffusion in the ion exchange resin phase, then the height equivalent to the theoretical plate is a function of the distribution coefficient and diminishes as λ' increases.

The dispersion (standard deviation) for the elution curve is given by the formula[21]:

$$\sigma = S\sqrt{L}\sqrt{1.64\,r\,(\lambda' + i)^2 + \frac{0.142\,\lambda'\,r^2\,u}{D_s} + \frac{0.266\,(\lambda')^2\,r^2\,u}{(1 - i)\,D_L\,(1 + 70\,r\,u)}} \tag{13}$$

where S is the area of the column cross section.

Its is well known that the diffusion coefficients increase with rise of temperature.

According to (12) and (13) the rise of temperature should bring about diminution of H as well as of σ.

Fig. 4 represents the experimentally determined relation between H and the distribution coefficient at different temperatures. It is easy to see from Fig. 4a that for a given value of the distribution coefficient and flow rate the height equivalent of the theoretical plate decreases, as the temperature increases. Simultaneously the value of H diminishes as the distribution coefficient increases, and this effect is particularly noticeable for small values of λ.

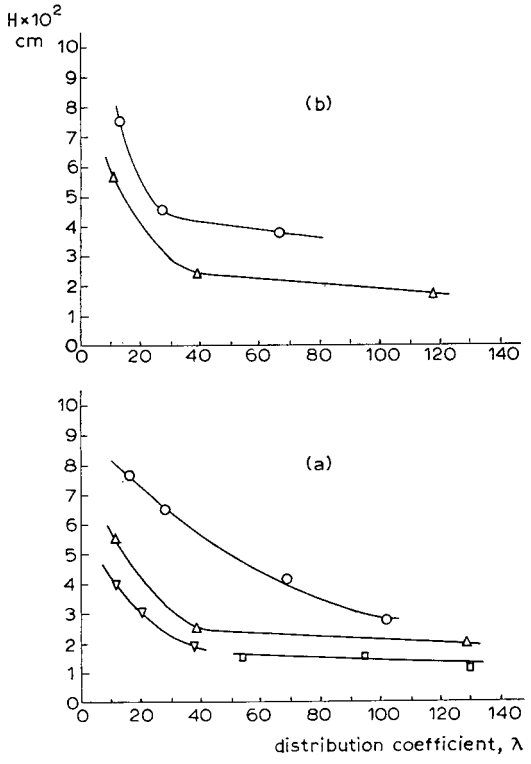


Fig. 4. Height equivalent to a theoretical plate as a function of distribution coefficient for various temperatures and flow rates. (a) —○—○— elution of Y, La, Ce and Pr; temp. 18°; —△—△— elution of Y, La and Pr; temp. 50°; —□—□— elution of Tb, Gd and Eu; temp. 55°; —▽—▽— elution of Lu, Sc and Ho; temp. 65°; u = 1.8–2.1·10^{-2} ml/cm^2/sec. (b) —○—○— elution of Y, La and Ce; u = 1.82·10^{-2} ml/cm^2/sec; —△—△— elution of Tm, La and Tb; u = 1.06·10^{-2} ml/cm^2/sec.; temp. 25°.

Fig. 4b shows the influence of the flow rate on the height equivalent of the theoretical plate. According to equation (12) as the flow rate decreases so does the value of H. All the data given above prove that the ion exchange reaction expressed by equation (1) is at least in part controlled by particle diffusion.

It can be shown from equation (10) that the greater the number of theoretical plates, the smaller the value of σ *i.e.* the narrower the peaks of separate components and the smaller their overlap.

The resolution of two components, R depends on the interval between the maxima as well as on the dispersions of the peaks themselves.

In gas chromatography the separation is generally deemed to be adequate when the interval between the maxima amounts to 4σ [22-23]; it is often assumed that the dispersion of both peaks is practically identical $\sigma_1 \cong \sigma_2 = \sigma$.

This problem has recently been considered in a more general way, the "resolution $N\sigma$" being taken as the criterion for the separation, the value of N being chosen according to the actual problem.

In ion exchange chromatography the assumption that the dispersions of neighbouring peaks are identical is generally far from being true. The resolution can thus be defined in general terms as follows:

$$R_n = \frac{U_{\max(2)} - U_{\max(1)}}{n(\sigma_1 + \sigma_2)} = \frac{\Delta U_{\max}}{n(\sigma_1 + \sigma_2)} \tag{14}$$

where n is an arbitrary number greater than 0.

In the present paper the separation of two components is taken as being complete if:

$$\Delta U_{\max} \geqslant 3(\sigma_1 + \sigma_2) \; i.e. \; R_3 \geqslant 1 \tag{15}$$

This means that after having divided the effluent into fractions at the point $U = U_{\max(1)} + 3\sigma_1$ at least 99.86 % of each of the components is in its appropriate fraction, and the impurity amounts to at most 0.14 % of the peak of the contiguous component.

It should be stressed, however, that for analytical purposes, *e.g.* for quantitative determinations effected directly from the elution curve by means of the previously described method[19], the condition that $R_2 \geqslant 1$, ($R_2 = 1.5\, R_3$) is in many cases sufficient.

Analysis of the elution curves

In Figs. 5–9 several experimental elution curves are shown which illustrate influence of temperature on the separation of rare earths. For each pair of peaks the separation factors α_1^2 are given (computed from formula (7)), as well as the resolutions R_3 computed from formula (14).

As has been shown above, as the temperature rises the number of the theoretical plates in the column increases. Simultaneously the separation factor changes on account of thermodynamic changes in the distribution coefficients of the components being separated.

For a certain limited range of temperature three cases are possible, *viz.*:

(a) α_1^2 increases as the temperature rises;

(b) α_1^2 remains practically constant;

(c) α_1^2 decreases as the temperature rises.

Case (a) is illustrated, among others, by the elution curves of the pairs Tb–Pm (Fig. 6), Eu–Pm (Fig. 7), Lu–Sc (Fig. 8) and Y–La (Fig. 9).

On account of the accumulation of the effects of the simultaneous increase of N and α_1^2 the resolution increases very rapidly as the temperature rises. In case (b) where the separation factor remains constant or changes only very slightly, the resolution increases far more slowly; *cf.* the elution curves of the pairs Gd–Eu (Fig. 5) and Ce–Pr (Fig. 9).

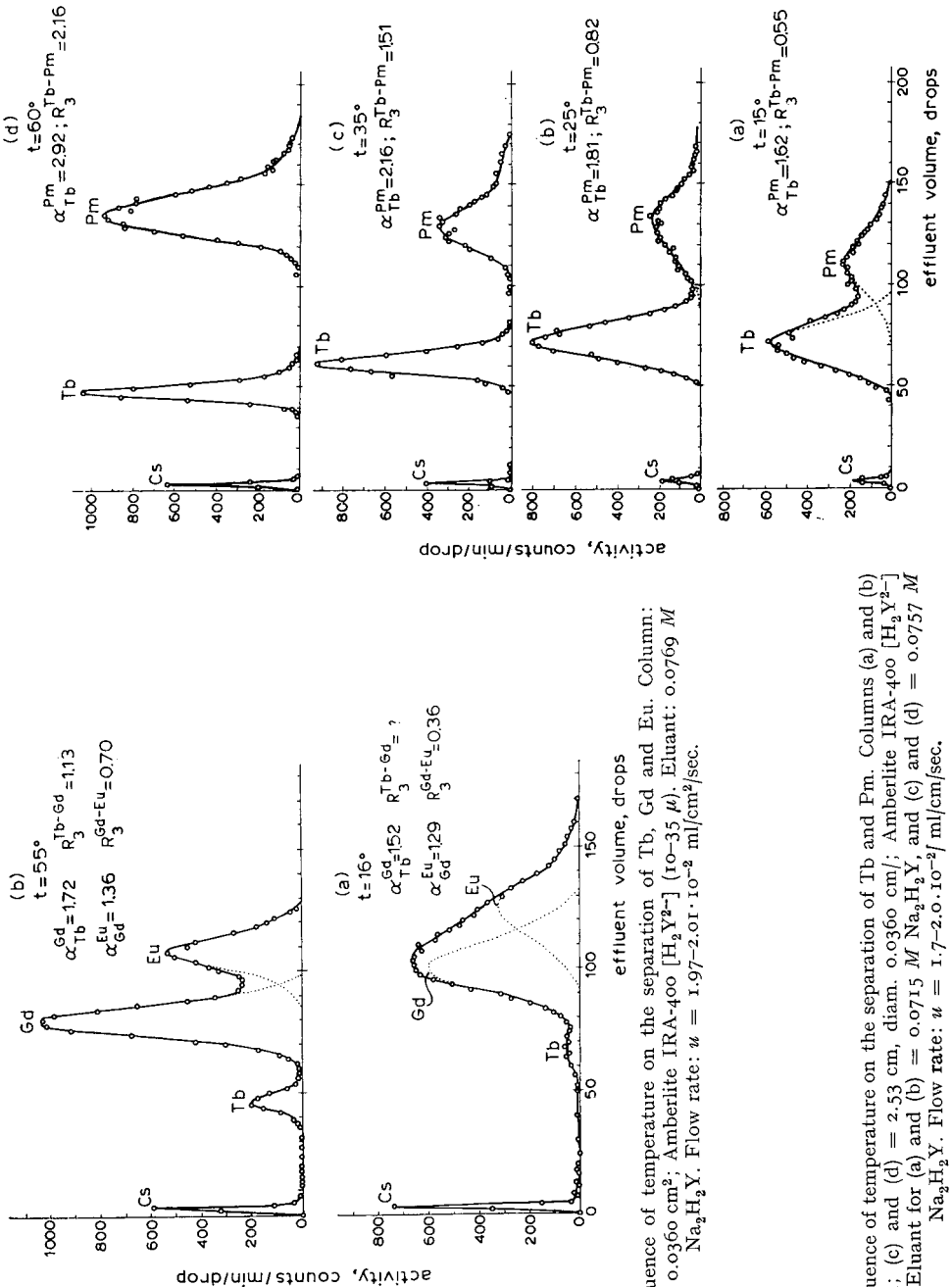


Fig. 6. Influence of temperature on the separation of Tb and Pm. Columns (a) and (b) = 2.50 cm; (c) and (d) = 2.53 cm, diam. 0.0360 cm/; Amberlite IRA-400 $[H_2Y^{2-}]$ (10–35 μ). Eluant for (a) and (b) = 0.0715 M Na_2H_2Y, and (c) and (d) = 0.0757 M Na_2H_2Y. Flow rate: u = 1.7–2.0·10^{-2} ml/cm/sec.

Fig. 5. Influence of temperature on the separation of Tb, Gd and Eu. Column: 2.50 cm × 0.0360 cm^2; Amberlite IRA-400 $[H_2Y^{2-}]$ (10–35 μ). Eluant: 0.0769 M Na_2H_2Y. Flow rate: u = 1.97–2.01·10^{-2} ml/cm^2/sec.

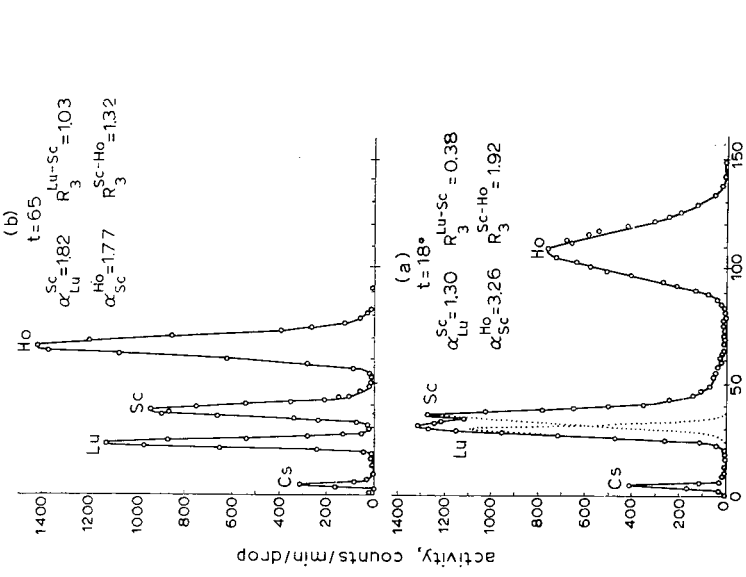


Fig. 8. Influence of temperature on the separation of Lu, Sc and Ho. Column: 4.65 cm × 0.0360 cm²; Amberlite IRA-400 [H_2Y^{2-}] (10–35 μ); Eluant: 0.0089 *M* Na_2H_2Y; Flow rate: u = 1.8–2.1 · 10⁻² ml/cm²/sec.

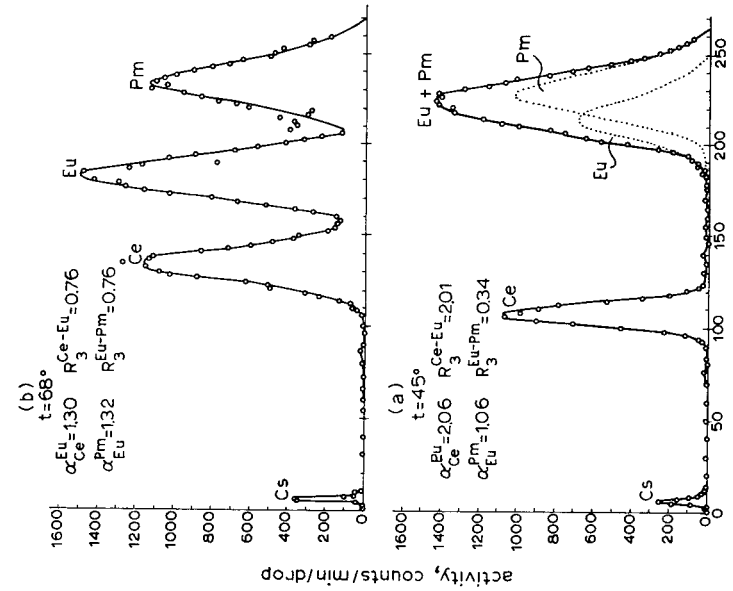


Fig. 7. Influence of temperature on the separation of Ce, Eu and Pm. Column: 2.90 cm × 0.0556 cm²; Amberlite IRA-400 [H_2Y^{2-}] (10–35 μ); Eluant: 0.0761 *M* Na_2H_2Y; Flow rate: u = 1.05–1.14 · 10⁻² ml/cm²/sec.

The last case (c) is perhaps the most interesting where changes of both factors affecting the final value of the resolution run in opposite directions. Depending on which of them prevails, the resolution can either decrease, *e.g.*, elution of the pairs Ce–Eu (Fig. 7) and Sc–Ho (Fig. 8), or increase, *e.g.* elution of the pair La–Ce (Fig. 9).

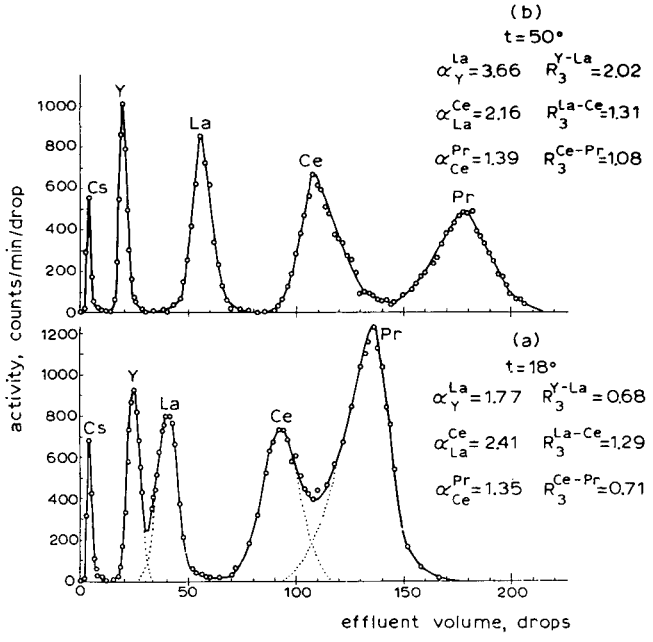


Fig. 9. Influence of temperature on the separation of Y, La, Ce and Pr. Column: 4.20 cm × 0.0360 cm²; Amberlite IRA-400 [H_2Y^{2-}] (10–35 μ); Eluant: 0.0447 *M* Na_2H_2Y; Flow rate: u = 1.82–1.88 · 10^{-2} ml/cm²/sec.

It, must however, be said that sometimes it is worth discarding very good resolution in order to considerably reduce the time of separation.

This is illustrated by the following example. At 18°C good resolution (R_3^{Sc-Ho} = 1.92) was obtained for the pair Sc–Ho, yet at the same time the separation of lutetium from scandium is unsatisfactory (see Fig. 8) and the time needed rather long. With an increase of temperature to 65°C, R_3^{Sc-Ho} = 1.32, the separation of scandium from holmium continues to be complete according to the assumed criterion (15), but on the other hand their separation time is nearly half. In addition to this, at 65°C the separation of lutetium from scandium is also complete (R_3^{Lu-Sc} = 1.03).

Other aspects of the changes of temperature

As has already been mentioned temperature influences also the separation time. The rate of movement of the band of the component down the column u_x, and strictly speaking the speed with which the peak maximum moves, is given by the formula[25]:

$$u_x = \frac{u}{\lambda' + i} \tag{16}$$

The speed of the movement of the band is approximately inversely proportional

to the distribution coefficient. Since the distribution coefficient is also a function of temperature hence the latter expresses (other conditions remaining unchanged) the time of separation.

The diminution of the dispersion of the peak as the temperature increases may also be important.

When using an ion exchange resin of very fine particles the flow of the liquid in the column is hindered considerably, and it can sometimes be very difficult to achieve an adequately high flow rate. The rise of temperature diminishes the hydraulic resistance and consequently a higher flow rate or the required flow rate at a lower pressure can be achieved.

As can be seen from Fig. 10 the pressure which must be applied for a given column in order to achieve the required flow rate decreases as the temperature rises and is also dependent upon the changes of viscosity of the water[26].

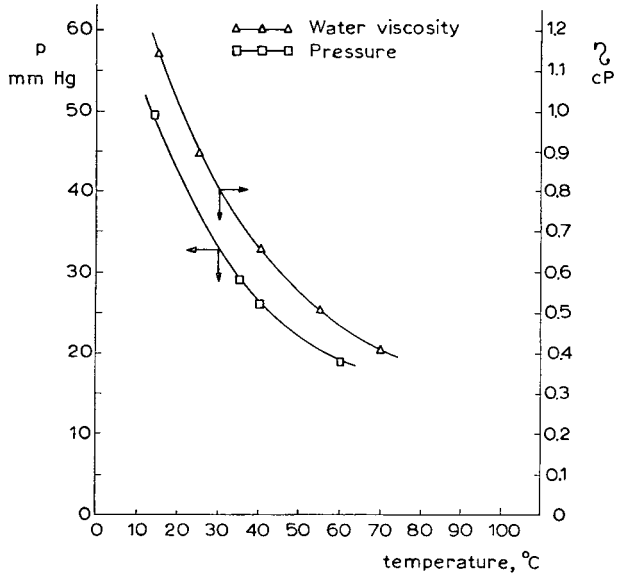


Fig. 10. Pressure which must be applied to obtain the desired flow rate, as a function of temperature. Column: 2.62 cm × 0.0360 cm²; Amberlite IRA-400 [H_2Y^{2-}] (10–35 μ); Eluant: 0.0754 M Na_2H_2Y; Flow rate: $u = 1.73–1.77 \cdot 10^{-2}$ ml/cm²/sec.

New possibilities of qualitative analysis

In a given chromatographic system the distribution coefficient is the characteristic feature of a substance and can help to identify it in a mixture. In ion exchange chromatography such a characteristic value can be the distribution coefficient, for a given concentration of the eluant, as well as the selectivity coefficient which in principle is independent from the concentration of the eluant; *cf.* equation (3). Conclusions drawn as to the presence or absence of a substance from λ or k values computed from the elution curve are not specific, since different substances can have identical or very similar distribution coefficients. Because of this, it has been suggested[27] that the

value of the selectivity coefficient should not be taken as a criterion of identification but its change as a function of temperature.

As has been shown in the present paper (Figs. 1 and 2) and in the literature[4,5,21] the $\log k$–$1/T$ curves have a different course for the seemingly similar substances. On account of this determination of selectivity coefficients from the elution curves for several discreet temperature intervals and comparison of these data with the previously determined $\log k$–$1/T$ curves of the substances of interest should be a more specific method. As far as the analysis of rare earths in the system discussed here is concerned this method allows identification of trace amounts of separate elements in a mixture, with a prevailing excess of other components. In this way it is possible to identify radioactive rare earths in such small amounts that the half-lives cannot be determined precisely[27].

ACKNOWLEDGEMENTS

The author is indebted to Prof. Dr. J. MINCZEWSKI for valuable discussions and observations and to Mrs. M. MARECKA for her help in carrying out the experiments.

SUMMARY

The influence of temperature on the anion exchange behaviour of the rare earth elements in the system: Amberlite IRA-400 [H_2Y^{2-}]–aq. solution of disodium ethylenediaminetetraacetate (Na_2H_2Y) has been investigated. In the system examined the change of temperature has a double effect: (1) the height equivalent to a theoretical plate decreases with a rise of temperature; (2) the distribution coefficients of the individual ions undergo changes according to the enthalpy values of the ion exchange reactions. As the enthalpies of the ion exchange reactions differ in magnitude and sign for different rare earths, the separation factors (ratio of distribution coefficients) may undergo considerable changes with a change of temperature. Both effects contribute to the final resolution value; for some elements the order of elution is even reversed.

New possibilities in qualitative analysis, resulting from the determination of the selectivity coefficient of an exchange reaction for a given ion at different temperatures, have been pointed out.

REFERENCES

1 B. H. KETELLE AND G. E. BOYD, *J. Am. Chem. Soc.*, 69 (1947) 2800.
2 G. R. CHOPPIN AND R. J. SILVA, *J. Inorg. Nucl. Chem.*, 3 (1956) 153.
3 O. D. BONNER AND R. R. PRUETT, *J. Phys. Chem.*, 63 (1959) 1417 and 1420.
4 K. A. KRAUS AND R. J. RARIDON, *J. Phys. Chem.*, 63 (1959) 1901.
5 K. A. KRAUS, R. J. RARIDON AND D. L. HOLCOMB, *J. Chromatog.*, 3 (1960) 178.
6 R. DYBCZYŃSKI, *Roczniki Chem.*, 37 (1963) 1411.
7 E. H. CRUICKSHANK AND P. MEARES, *Trans. Faraday Soc.*, 53 (1957) 1289.
8 R. DYBCZYŃSKI, *Mitteilungsblatt der Chemischen Gesellschaft in der DDR*, Sonderheft 1960, Analytische Chemie, p. 393–401.
9 J. MINCZEWSKI AND R. DYBCZYŃSKI, *Chem. Anal. (Warsaw)*, 6 (1961) 275.
10 J. MINCZEWSKI AND R. DYBCZYŃSKI, *J. Chromatog.*, 7 (1962) 98.
11 J. MINCZEWSKI AND R. DYBCZYŃSKI, *J. Chromatog.*, 7 (1962) 568.
12 W. J. BLAEDEL AND H. T. KNIGHT, *Anal. Chem.*, 26 (1954) 741.
13 R. M. WHEATON AND W. C. BAUMAN, *Ind. Eng. Chem.*, 43 (1951) 1088.
14 R. DYBCZYŃSKI, *Przemysł Chem.*, 38 (1959) 216.
15 F. NELSON, *J. Am. Chem. Soc.*, 77 (1955) 813.

[16] E. GLUECKAUF, *Trans. Faraday Soc.*, 51 (1955) 34.
[17] F. W. CORNISH, *Analyst*, 83 (1958) 634.
[18] S. W. MAYER AND E. R. TOMPKINS, *J. Am. Chem. Soc.*, 69 (1947) 2866.
[19] J. MINCZEWSKI AND R. DYBCZYŃSKI, *Chem. Anal. (Warsaw)*, 6 (1961) 725.
[20] E. GLUECKAUF, *Ion Exchange and its Applications*, Society of Chemical Industry, London, 1958.
[21] P. B. HAMILTON, D. C. BOGUE AND R. A. ANDERSON, *Anal. Chem.*, 32 (1960) 1782.
[22] *Gas chromatography*, D. H. DESTY, Editor, Butterworths Scientific Publications, London, 1958, p. xi.
[23] D. AMBROSE, A. T. JAMES, A. I. M. KEULEMANS, E. KOVÁTS, H. RÖCK, C. ROUIT AND F. H. STROSS, *Pure and Applied Chemistry*, 1 (1960) 169.
[24] H. STRICKLER AND E. SZ. KOVÁTS, *J. Chromatog.*, 8 (1962) 289.
[25] F. HELFFERICH, *Ion Exchange*, Mc. Graw-Hill Book Company Inc., New York, 1962, p. 455.
[26] *Kalendarz Chemiczny*, Part. I, PWT, Warsaw, 1954, p. 280.
[27] R. DYBCZYŃSKI, *Anal. Chim. Acta*, 29 (1963) 369.

Short Communication

The isolation and purification of aliphatic sulphoxides on a strongly acidic cationic exchanger

The preparation of pure sulphoxides, for example for various physico-chemical and kinetic measurements, is in general rather difficult. Even though advantageous methods are described for the oxidation of sulphides to sulphoxides[1], the crude product is always contaminated with the starting material and with the further oxidation product, the sulphone. The separation of liquid sulphoxides from all byproducts by simple distillation is never completely successful, owing to their thermolability. In many cases sulphoxides and sulphones form mixed crystals.

Adsorption on a strongly acidic cationic exchanger Dowex 50 proved suitable for the selective separation of aliphatic sulphoxides. The exchanger was used in the H-cycle and before use it was washed successively with distilled water, ethanol and finally with benzene. A benzene solution of the crude sulphoxide was poured on to the exchanger gel; only one tenth to fifth of the over-all column capacity was used. The column was washed with another portion of benzene. The sulphoxide was desorbed with ethanol, which proved most suitable. The pure sulphoxide was recovered by evaporating the ethanol solution in vacuum at low temperature and vacuum distilling at 10^{-1} torr. The sulphoxide content was followed by iodometric titration[2]. The degree of purity reached was as follows: methyl *n*-butyl, 99.9 %; ethyl *n*-butyl, 99.7 %; *n*-propyl *n*-butyl, 100 %; isopropyl *n*-butyl, 99.5 %; isobutyl *n*-butyl, 99.8 %; *sec.*-butyl *n*-butyl, 99.1 %.

Aromatic sulphoxides and dibenzyl sulphoxide are not adsorbed from benzene solution on to Dowex 50.

The interaction of the sulphinyl group of the sulphoxide with the sulphonyl group of the exchanger is probably due to hydrogen bonding. The sulphoxide-hydrogen affinity has been studied carefully in the past[3]. A detailed study of the phenomenon described above, from the theoretical point of view, will be reported in a separate paper.

Institute of Organic Chemistry of the Charles University, Prague (Czechoslovakia)

V. HORÁK
J. PECKA

[1] N. J. LEONARD AND C. R. JOHNSON, *J. Org. Chem.*, 27 (1962) 282.
[2] F. JANČÍK AND J. KÖRBL, *Ceskoslov. Farm.*, 11 (1962) 305.
[3] D. BARNARD, J. M. FABIAN AND H. P. KOCH, *J. Chem. Soc.*, (1949) 2442.

Received November 25th, 1963

Notes

A reaction occurring between benzoin and quinone on a gas chromatographic column

During the course of a systematic study of the behaviour of benzoin and various substituted benzoins on gas chromatographic columns (to be published at a later date), a mixture of benzoin and *p*-benzoquinone in methyl ethyl ketone was injected into a column. The peaks that were detected besides the solvent consisted of hydroquinone, deoxybenzoin, and benzil. Quinone itself was never detected even after 23 min at 182°. (This in itself is a little surprising as quinone is relatively volatile.) Mixture solutions of varying concentrations were used and the sample size in all cases was 40 μl. The hydroquinone peak appeared on the tail of the solvent peak and was sharp and symmetrical allowing for its position. Authentic samples of hydroquinone had retention times slightly shorter, 1.85–1.90 min, than the produced hydroquinone, 1.95–2.05 min.

Benzoin itself on injection into a column gives only deoxybenzoin and benzil with no benzoin at all coming out. At 182° the molar ratio benzil/deoxybenzoin is 2.0 for an injection of only benzoin. This ratio increases with the amount of quinone present. No reaction occurred between the benzoin and quinone in the cold solution. However, on heating approximately 1/1 molar mixture of benzoin and quinone to 190° in a sealed evacuated tube (10^{-3} mm Hg) a reaction was seen with the mixture turning black. The IR spectrum of a solution of the product in methyl ethyl ketone gave a peak at 828 cm^{-1} ascribable to neither benzoin, quinone or solvent but also given by a solution of hydroquinone. Pure quinone itself, and pure benzoin itself under the same conditions, underwent no changes. Visual inspection of the column after injections of quinone-benzoin mixtures showed progressive blackening of the first few cm, with most of the blackening on the glass wool plug immediately above the packing. After injection of a sample containing quinone it took two successive injections of benzoin solution for the benzil–deoxybenzoin ratio to return to its usual value for that column.

Experimental

The gas chromatograph used was Burrel model K-1 with its new type thermal conductivity detector at 200°.The column used was 250 cm long and filled with 20 % Dow Corning high vacuum grease on 80–100 mesh Chromosorb W. The flow rate in all cases was 50 ml/min of helium. The column temperature except where otherwise noted was 182°, and sample size 40 μl, methyl ethyl ketone as solvent. The hydroquinone, deoxybenzoin, and benzil were all identified by trapping the respective peaks and comparing their infrared spectra with those of authentic samples. The quinone used was obtained by the method of MacCoy[5]. The benzoin was from Matheson, Coleman and Bell, m.p. 134–135°. The hydroquinone was B.D.H. Cert. m.p. 171–173°. Blank runs were made of both benzoin and quinone. In some cases to be absolutely sure the quinone was further purified by vacuum sublimation.

Results and discussion

As seen in Table I the amount of hydroquinone produced is almost linear with that of injected quinone but shows a slight tendency to level off which would be expected at a constant benzoin concentration. The general increase in the benzil/deoxybenzoin ratio would also be expected, the quinone would be competing with the benzoin for the

TABLE I

AMOUNTS OF PRODUCTS AFTER CHROMATOGRAPHY

Benzoin concn. M	*Quinone concn. M*	*Quinone injected μmoles*	*Hydroquinone out μmoles*	*Benzil/ deoxybenzoin*
0.114	0.774	31.9	5.49	15.6
0.114	0.472	18.9	4.06	4.58
0.114	0.254	10.19	2.63	3.40
0.114	0.217	8.7	1.95	4.94
0.114	0.119	4.76	0.983	3.54
0.114	0.0	0.0	0.0	2.0

acceptance of the hydrogen. The lowest column temperature used was 158°, at which temperature at least 50 % as much hydroquinone is produced as at 182°.

Reactions occurring in the course of gas chromatography are well known, for example one can obtain catalytic isomerization of terpenes as well as catalytic and pure thermal decomposition of oxygenated terpenes[1,2]. Dehydration, deacetylation, and *cis–trans* isomerization have all been observed in the GLC of the methyl esters of fatty acids[3]. These changes were primarily thermal but to some extent metal catalysed.

The reaction between benzoin and quinone is definitely thermal as it occurred on the simple heating of the two. On the other hand, the self reaction of benzoin is definitely catalysed by the column. The reaction between the benzoin and quinone is of interest as reactions involving hydrogen transfer during GLC are unusual and also GLC provides a convenient way to study this reaction[4].

Acknowledgement

Grateful acknowledgement is made for financial support from the National Research Council of Canada.

St. Francis Xavier University,
Antigonish, Nova Scotia (Canada)

D. L. BUNBURY
M. S. OSYANY

[1] D. H. DESTY, *Gas Chromatography*, Academic Press, 1958, p. 214-215.
[2] E. A. DAY AND P. H. MILLER, *Anal. Chem.*, 34 (1962) 869.
[3] L. J. MORRIS, R. T. HOLMAN AND KRISTER-FONTELL, *J. Lipid Res.* 1 (1959) 412.
[4] S. Z. ROGINSKII, M. I. YANOVSKII AND G. A. GAZIEV, *Dokl. Akad. Nauk SSSR*, 140 (1961) 1125.
[5] H. N. MACCOY, *J. Chem. Educ.*, 14 (1939) 494.

Received August 12th, 1963

The use of amino acids as stationary phase in gas chromatography

Part 1. Monosodium L-glutamate

The analysis of amino acids is an essential part of peptide and protein studies. Not only is it of importance for the determination of the overall composition of these biological materials but it has acquired added importance in studies of amino acid sequence in peptide chains. Present available methods in sequence studies, such as paper chromatography, paper electrophoresis and ion exchange chromatography are all time-consuming procedures. On the contrary, gas chromatographic analysis, which needs neither a large amount of sample nor a long operating time, might be useful in studies on proteins. Several attempts to employ gas-liquid chromatography for amino acids have been made[1-7].

JOHNSON AND MEISTER[8] have recently reported on the chromatography of a number of N-acetylamino acid *n*-amyl esters using 2- to 8-foot column packed with Chromosorb W coated with 0.5% to 5% polyethylene glycol (Carbowax 1540 or 6000). We investigated the gas chromatographic analysis of N-acetylamino acid *n*-amyl esters on 2- to 3-meter column packed with Carbowax 1540 coated onto Chromosorb according to JOHNSON *et al.*, and have found that this method was unfortunately time-consuming for the elution, and moreover, it gave unsatisfactory resolution of these derivates.

In the present note, the unique application of an optically active amino acid as selective stationary phase is described for the rapid and effective separation of amino acid derivatives. 10%-monosodium L-glutamate coated onto sodium chloride (commercial name, "Aji-Shio", Ajinomoto Co.,) was found to give satisfactory results. Furthermore, we are trying to extend the application of optically active amino acids to the gas chromatographic resolution of optical antipodes.

Apparatus

A Perkin-Elmer Model 154-D gas chromatograph equipped with a hydrogen flame-ionization detector was used. The carrier gas was nitrogen. A 50 cm to 2 m U-shaped stainless-steel column $^1/_4$ in. external diam. was packed with monosodium L-glutamate coated onto sodium chloride, or with monosodium L-glutamate or L-glutamic acid when used without a support medium.

Materials

N-acetylamino acid esters were prepared with HCl–alcohol and acetic anhydride. Derivatives were analysed by infrared spectroscopy. Fatty acids and fatty acid methyl esters made by Tokyo Kasei Co. were used without further purification.

Results

The gas chromatograms of some amino acid derivatives and fatty acids on "Aji-Shio" are given in Figs. 1 and 2, respectively. All solutes were eluted very rapidly and moreover, the resolution of each peak was very high. In the above experiments a very wide range of particle sizes of commercial "Aji-Shio" was used. In Figs. 3 and 4 (the chromatograms of amino acid derivatives and fatty acid methyl esters, respectively), sieved "Aji-Shio" (50–100 mesh) was used. As can be seen from Figs. 1–4, the reten-

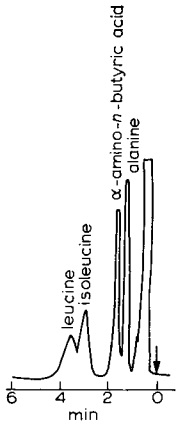


Fig. 1. Chromatogram of N-acetyl-*n*-amyl esters of 4 amino acids on a 2 m column packed with commercial "Aji-Shio" (10% monosodium glutamate coated on to NaCl). Temperature: 159°; carrier gas: nitrogen; Flow rate: 66ml/min.

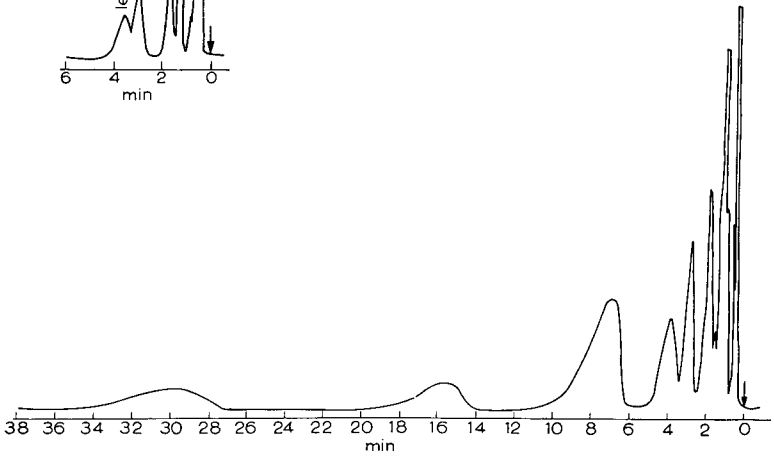


Fig. 2. Chromatogram of a mixture of C_1–C_8 acids on commercial "Aji-Shio". Column length: 1 m; Temperature: 134°; carrier gas: nitrogen; Flow rate: 48 ml/min.

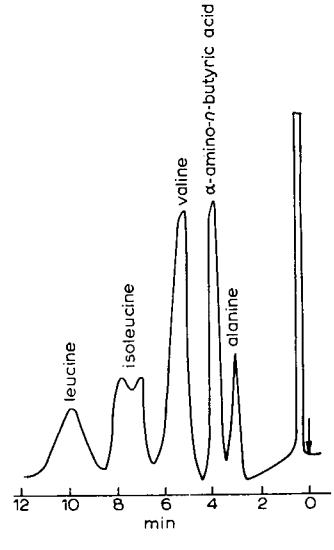


Fig. 3. Separation of N-acetyl-*n*-amyl esters of 5 amino acids on a 2 m column containing 10% monosodium glutamate on NaCl (50–100 mesh) at 155° and 45 ml of N_2 per minute.

Fig. 4. Separation of methyl esters of several fatty acids at 155° and 53 ml of N_2 per minute on a 2 m column containing 10% monosodium glutamate on NaCl (50–100 mesh).

Fig. 5. Chromatogram of N-acetyl-*n*-amyl esters of 4 amino acids on a 50 cm column packed with monosodium glutamate (50-100 mesh); no NaCl used as support. Temperature: 159°; Flow rate: 40 ml/min of N_2.

Fig. 6. Chromatogram of a mixture of C_1–C_8 acids on a 50 cm column packed with monosodium glutamate (50-100 mesh); no NaCl used as support. Temperature: 143°; Flow rate: N_2 53 ml/min.

Fig. 7. Chromatogram of methyl esters of fatty acids on a 50 cm column packed with glutamic acid (50-100 mesh) at 145° and 53 ml/min of N_2.

tion times of all components were very short and were the same for sieved "Aji-Shio" as for the unsieved material, but the sieved material gave higher resolution. DL-Isoleucine showed two incompletely resolved peaks. We confirmed that these two peaks correspond to D-alloisoleucine and L-isoleucine, respectively.

The chromatograms of some derivatives of amino acids and fatty acids on 50-100 mesh monosodium L-glutamate without support are shown in Figs. 5 and 6; this gave short retention times but poor resolution. It has been suggested that a satisfactory resolution could be obtained under suitable chromatographic conditions of column length, column temperature, particle size, etc. In Fig. 7, a chromatogram of fatty acid methyl esters on 50–100 mesh L-glutamic acid without a support, the same result was obtained as that when monosodium L-glutamate was used.

Hitherto, for the complete separation of active-amyl alcohol and iso-amyl alcohol a very long column was required and then the retention time was long. It was found that "Aji-Shio" is useful for the separation of these alcohols as is shown in Fig. 8.

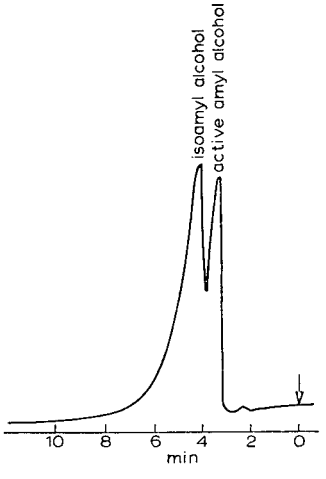


Fig. 8. Separation of active-amyl alcohol and iso-amyl alcohol on a 1 m column containing 10% monosodium glutamate on NaCl (50–100 mesh) at 48° and 48 ml/min of N_2.

Monosodium L-glutamate is stable at high column temperatures (up to 210°) and this fact permits its use in the separation of high-boiling compounds without the danger of decomposition of the stationary phase.

The author wishes to thank MR. S. KUBOTA for his assistance in the gas chromatographic experiments.

Central Research Laboratory, Ajinomoto Co., Inc., Kawasaki, Kanagawa (Japan)

ASAO MURAI
YOKO TACHIKAWA

1 I. R. HUNTER, K. P. DIMICK AND J. W. CORSE, *Chem. & Ind. (London)*, (1956) 294.
2 A. ZLATKIS, J. F. ORÓ AND A. P. KIMBALL, *Anal. Chem.*, 32 (1960) 162.
3 M. BIER AND C. TEITELBAUM, *Ann. N.Y.Acad.Sci.*, 72 (1959) 641.
4 E. BAYER, K. H. REUTHER AND F. BORN, *Angew. Chem.*, 69 (1957) 640.
5 C. G. YOUNGS, *Anal. Chem.*, 31 (1959) 1019.
6 H. A. SAROFF AND A. KARMEN, *Anal. Biochem.*, 1 (1960) 344.
7 A. LIBERTI, *Gas Chromatography 1958*, D. H. DESTY, ed., Academic Press, New York, 1958, p. 341.
8 D. E. JOHNSON, S. J. SCOTT AND A. MEISTER, *Anal. Chem.* 33 (1961) 669.

Received July 17th, 1963

Gas chromatographic behaviour of normal aliphatic ester isomers

Throughout the gas chromatographic identification of volatile tropical fruit flavours in this laboratory the prediction of retention times from known data has become a necessity. With fruit flavours one is faced with such a wide array of possible chemical compounds that the synthesis of these to provide retention data is impracticable.

Much use has been made of plots of logarithm of retention time *versus* number of carbon atoms for an homologous series[1] but this relationship is of restricted application. As esters comprise an important fraction of known tropical fruit flavours[2,3] an examination of isomeric esters was initiated. This revealed regular relationships between retention times and boiling points of normal aliphatic esters containing a constant number of carbon atoms, *e.g.* methyl *n*-caproate, ethyl *n*-valerate, *n*-propyl *n*-butyrate etc. for a group containing seven carbon atoms. These relationships have proved useful in predicting retention times unobtainable by other means.

Experimental

Apparatus

A Perkin Elmer Model 154D Vapor Fractometer fitted with a thermistor bead detector and a 2 m × $^1/_4$ in. o.d. stainless steel column packed with dinonyl phthalate on kieselguhr was used. The instrument was operated at a column temperature of 168° and a flow rate of 46 ml of helium/min.

Reference compounds

Normal aliphatic esters containing from four to nine carbon atoms were prepared by the usual synthetic procedures. Fractional distillation was carried out where the product was grossly impure. These esters will fall into six constant number of carbon atoms groups similar to that previously illustrated and hereafter are referred to as "ester groups".

Results

For each ester group retention times under the conditions previously described were obtained and the logarithm plotted against the boiling point. In each case a straight line was obtained with only two instances of a marked deviation (Fig. 1E and F). Here it is believed accurate determination of the boiling points would reveal closer agreement.

Discussion

Mathematical extension

By the following simple mathematical extension the usefulness of the above relationship can be expanded:

From Fig. 1:

$$\frac{\log R_T}{\text{b.p.}} = k \text{ (a constant)}$$

Suppose the plots shown in Fig. 2 were made for each ester group (a_1, a_2 etc. and b_1, b_2 etc. are equal to the boiling points and log R_T values respectively).

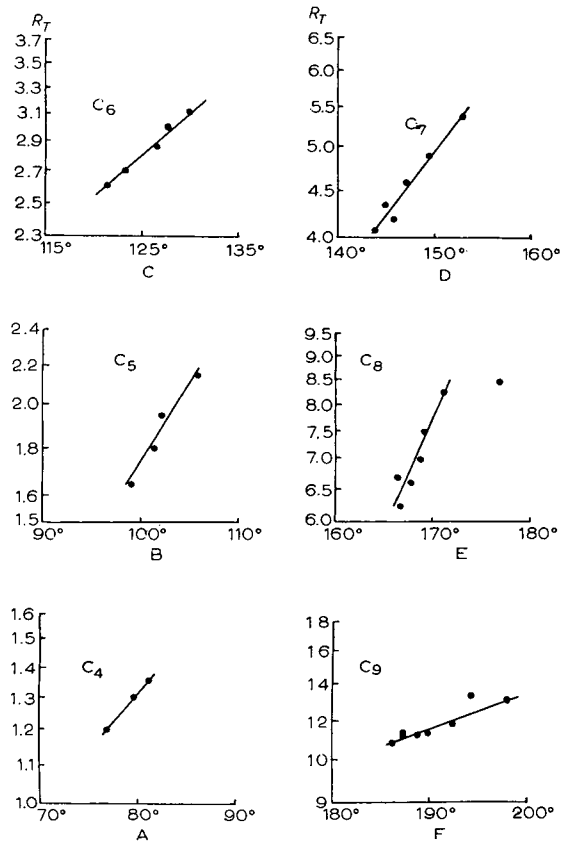


Fig. 1. Plots of logarithm of retention time (in min) *versus* boiling point (°C) for each ester group.

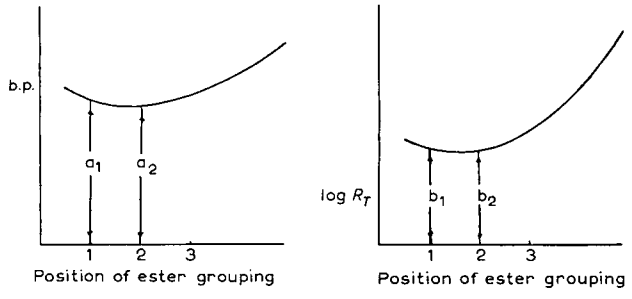


Fig. 2. Hypothetical plots of boiling point and logarithm of retention time *versus* position of the ester grouping.

Now:

$$\frac{a_1}{b_1} = \frac{a_2}{b_2} \cdots \frac{a_n}{b_n} = k$$

Thus the curves in Fig. 2 must have the same shape.

This means the curves produced by a particular ester group will be geometrically superimposable. This can be done by taking one graphic difference in boiling points as being equivalent to the corresponding graphic difference in log R_T values and altering the other values correspondingly.

Fig. 3 shows boiling point plots superimposed on retention time plots for each ester group. Substantial agreement is shown in each case.

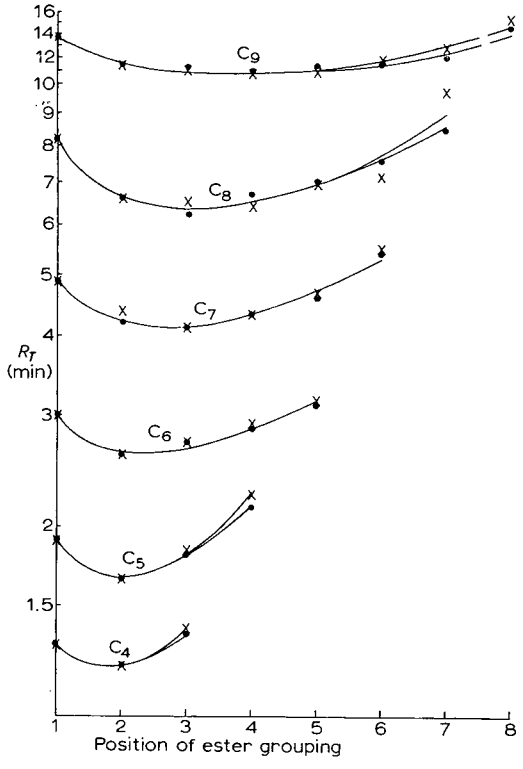


Fig. 3. Plots of boiling point *versus* position of the ester grouping (as number of C atoms in the alcohol portion of the ester), superimposed upon similar retention time plots. (×) retention times; (●) superimposed boiling points.

Application

There are two methods of application:

(i) if the retention times and precise boiling points of two esters in a group are known, a plot corresponding to those shown in Fig. 1 can be made. Thus knowledge of the boiling points of other members will enable direct reading off of the retention times.

(ii) with this method, knowledge of all the boiling points is not required but enough must be known to construct the curve for boiling points analogous to that shown in Fig. 2. Two retention times must also be known to enable the construction of the remainder of the retention time curve by geometric superimposition of the boiling point curve. From this construction retention times may be read directly for any member of the group.

The two methods depend upon precise measurement of retention times and an accurate knowledge of boiling points. In some cases where the boiling points are very close the retention times of a number of compounds may be indistinguishable.

Conclusions

Gas chromatographic examination of normal aliphatic ester isomers has revealed two relationships useful in the prediction of retention times. It is believed that these relationships can be extended not only to other similar series of esters but to any series of compounds which maintains a basic structure about which or within which a group of constant size is shifted in a regular fashion.

Food Preservation Research Laboratory,
Department of Agriculture and Stock,
Brisbane, Q'ld. (Australia)

D. W. CONNELL

[1] G. J. PIEROTTI, C. H. DEAL, E. L. DERR AND P. E. PORTER, *J. Am. Chem. Soc.*, 78 (1956) 2989.
[2] A. J. HAAGEN-SMITH, J. G. KIRCHNER, A. N. PRATER AND C. L. DEASY, *J. Am. Chem. Soc.*, 67 (1946) 1646.
[3] P. ISSENBERG AND E. L. WICK, *J. Agr. Food Chem.*, 11 (1963) 2.

Received August 19th, 1963

R_F values of some catecholamines, precursors and metabolites

In an effort to find a two-dimensional paper chromatographic system suitable for the radioautography of catecholamine metabolites of tyrosine and related compounds, the R_F(s) of various commercially obtained standards were determined in a number of solvent systems. All experiments were done with Whatman No. 1 paper and with diazotized sulfanilic acid and/or ninhydrin as the detecting reagents. Descending chromatography was used except where otherwise specified. The data are tabulated in Tables I and II. The preferred bidimensional system was methanol–butanol–benzene–water (4:3:2:1), run with the grain of the paper in the long direction, followed by *n*-butanol saturated with 1 *N* hydrochloric acid, a combination which gave reasonable resolution and excellently formed spots.

Certain of these solvents were also tried in solvent reversals such as used by WEISS AND ROSSI[1]. The results are given in Table III, along with the corresponding values

TABLE

R_F VALUES (× 100) OF SOME CATECHOLAMINES,

	S_1	S_2	S_3	S_4	S_5	S_6[t]	S_6[t*]	S_7	S_8[t]	S_9[t]
Tyrosine	55	76	47	49[t]	46	56	43	67	65	22
Tyrosine-O-sulfate	—	—	—	—	—	—	—	—	—	—
Tyramine	74	78	—	80	66	72	61	85	79	54
p-Hydroxyphenylacetic acid	65	79	65	66	65	72	60	80	80	—
3,4-Dihydroxyphenylalanine	47	68	40	54	36	46	37	59	62	5
3,4-Dihydroxyphenylacetic acid	59	67	57	60	57	66	56	76	80	—
Dopamine	71	79	66	73	58	66	54	75	72	36
Noradrenaline	68	78	61	71	54	65	55	77	65	18
Adrenaline	75	79	70	74	63	71	—	82	73	16
Metanephrine	72	76	70	73	64	70	—	80	81	—
Normetanephrine	72	80	68	73	62	68	—	81	72	—
3-Methoxytyramine	72	79	70	74	66	73	60	81	80	42

t = tendency to streaking; * = ascending
S_1 = *n*-butanol–pyridine–0.2 *N* sodium acetate (1:1:1); $13^1/_2$ h.
S_2 = *n*-butanol–pyridine–1 *M* sodium acetate (1:1:2); aqueous phase; $9^1/_2$ h.
S_3 = *n*-butanol–pyridine (1:1) saturated with 1 *M* sodium acetate; $14^1/_2$ h.
S_4 = *n*-butanol–pyridine–water (1:1:1); 13 h.
S_5 = benzene–methanol–butanol–pyridine–water (1:2:1:1:1); $9^1/_2$ h.
S_6 = methanol–water–pyridine (20:5:1); 11 h descending, 24 h ascending.
S_7 = toluene–ethyl acetate–pyridine–water–methanol (1:1:1:1:1); 7 h.
S_8 = toluene–ethyl acetate–methanol–water (1:1:1:1); aqueous phase; 7 h.
S_9 = water–saturated methyl ethyl ketone; 9 h.
S_{10} = *n*-butanol–ethanol–water (2:1:1); $13^1/_2$ h.
S_{11} = methanol–*n*-butanol–benzene–water (4:4:4:1); 8 h.
S_{12} = methanol–*n*-butanol–benzene–water (2:1:1:1); $15^1/_2$ h.
S_{13} = methanol–*n*-butanol–benzene–water (4:3:2:1); 12 h descending, 15–23 h ascending.
S_{14} = toluene–ethyl acetate–methanol–0.1 *N* HCl (1:1:1:1); 7 h.
S_{15} = methanol–amyl alcohol–benzene–2 *N* HCl (37:17.5:35:12.5); $9^1/_2$ h.
S_{16} = *n*-butanol saturated with 1 *N* HCl; 24 h descending, $26^1/_2$ h ascending.
S_{17} = *tert.*-butanol–acetone–formic acid–water (160:160:1:39); $9^1/_2$ h.
S_{18} = chloroform–acetic acid–water (2:1:1); aqueous phase; 7 h.
S_{19} = *n*-butanol–acetic acid–water (4:1:1); 10 h.
S_{20} = *tert.*-butanol–acetone–propionic acid–water (160:160:1:39); $8^1/_2$ h.
S_{21} = benzene–propionic acid–water (2:1:1); $8^1/_3$ h.

I

PRECURSORS AND METABOLITES

S_9*	S_{10}	S_{11}	S_{12}	S_{13}	S_{13}*	S_{14}	S_{15}	S_{16}	S_{16}*	S_{17}t	S_{17}t*	S_{18}	S_{19}	S_{20}	S_{21}
12	43	—	49	45	42	78	61	38	30	20	25	81	35	14	83
—	—	—	15	10	—	—	—	6	—	—	—	9	5	—	—
10	60	36	72	66	65	80	58	42	38	55	55	90	62	—	92
95	73	—	79	64	72	81	67	84	86	86	94	88	90	87	87
2	23	—	37	21	20	75	51	19	18	8	6	80	21	6	83
92	61	—	68	54	62	82	69	67	75	85	—	83	80	80	92
7	47	23	61	51	49	76	48	22	20	38	37	86	42	38	88
6	40	16	55	42	43	72	40	13	13	27	29	83	30	28	87
—	48	21	61	58	55	76	45	17	—	32	—	88	39	34	90
—	52	34	69	68	—	80	59	27	—	41	—	91	51	35	93
—	50	25	65	56	—	79	50	22	—	35	—	87	45	30	90
8	57	34	70	61	62	80	57	33	33	43	42	90	58	41	92

TABLE II

R_F VALUES (× 100) OF SOME ADDITIONAL TYROSINE AND CATECHOLAMINE METABOLITES

Compound	S_{12}	S_{13}	S_{16}	S_{19}
N-Acetyltyrosine	85	82t	52	81
O-Acetyltyrosine	64	49	33	47
3-Methoxytyrosine	63	53	26	37
Homovanillic acid	91	67	61	84
3-Methoxy-4-hydroxy-mandelic acid	59	39	48	69
3,4-Dihydroxymandelic acid	50	30	46	54

S_{12} = methanol–*n*-butanol–benzene–water (2:1:1:1); $15^1/_2$ h.
S_{13} = methanol–*n*-butanol–benzene–water (4:3:2:1); 12 h descending, 15–23 h ascending.
S_{16} = *n*-butanol saturated with 1 *N* HCl; 24 h descending, $26^1/_2$ h ascending.
S_{19} = *n*-butanol–acetic acid–water (4:1:1); 10 h.

TABLE III

COMPARISON OF CALCULATED AND EXPERIMENTAL R_N VALUES FOR SOME SOLVENT REVERSALS*

Compound	Experimental and (theoretical) R_N values (× 100)							
	S_7 followed by S_{19}			S_1 followed by S_{19}	S_7 followed by S_{20}	S_{19} followed by S_{12}	S_{12} followed by S_{18}	Phenol–HCl** followed by S_{19}
	$x^* = 0.23$	$x = 0.16$	$x = 0.30$	$x = 0.12$	$x = 0.49$	$x = -0.24$	$x = -0.19$	$x = 0.20$
Dopamine	25 (34)	48 (37)	35 (31)	30 (36)	50 (27)	35 (31)	63 (43)	31 (21)
Dopa	33 (42)	55 (43)	48 (40)	34 (34)	65 (52)	(22)	55 (33)	16 (18)
Adrenaline	31 (41)	56 (44)	44 (38)	38 (41)	51 (37)	33 (30)	67 (45)	40 (28)
Noradrenaline	34 (47)	53 (49)	48 (45)	37 (44)	60 (42)	28 (27)	66 (44)	20 (17)
Tyrosine	19 (36)	44 (38)	33 (33)	27 (31)	62 (51)	34 (29)	68 (38)	—
Tyramine	11 (18)	28 (22)	16 (14)	19 (21)	40	38 (35)	56 (39)	—
p-Hydroxyphenyl-acetic acid	−6 (−13)	1 (−6)	(−19)	−11 (−4)	−21 (−32)	36 (38)	33 (25)	73
3,4-Dihydroxyphenyl-acetic acid	−3 (−3)	12 (2)	(−9)	8 (−2)	(−24)	40 (42)	40 (29)	18 (−2)
Metanephrine	15 (27)	34 (31)	22 (24)	23 (29)	45 (35)	35 (32)	(43)	45 (31)
Normetanephrine	19 (34)	41 (37)	28 (31)	30 (34)	51 (42)	33 (31)	65 (44)	43 (28)
3-Methoxytyramine	10 (21)	29 (25)	(17)	18 (23)	45 (28)	(34)	(40)	39 (21)

* R_N, as defined by WEISS *et al.*[2], is the ratio of the distance of the spot from the initial starting line to the distance travelled by the first solvent; x is the solvent distance ratio. The calculated R_N is given in parentheses following the experimental R_N. For solvents see Table I.

** 88% aqueous phenol in an atmosphere of 1 *N* HCl; R_F data given by WEISS *et al.*[2] for this solvent used in calculating R_N.

calculated as indicated by WEISS *et al.*[2]. The spots produced by this technique are sharper than those obtained with ordinary one- or two-dimensional chromatography.

Acknowledgements

This research was supported by Federal-Provincial Mental Health Grant No. 609-5-141, the National Science Foundation, and the Canadian Mental Health Association.

Kinsmen Laboratory of Neurological Research, EDITH G. MCGEER
The University of British Columbia, Vancouver, B.C. (Canada) WILLIAM H. CLARK

[1] B. WEISS AND G. V. ROSSI, *Nature*, 195 (1962) 178.
[2] B. WEISS, G. V. ROSSI AND L. A. REBER, *Nature*, 197 (1963) 280.

Received August 27th, 1963

Temperature control in paper chromatography

In order to overcome the considerable variations in R_F values of paper chromatograms and the even more marked effects on reverse phase systems that occur with temperature variation, it is usual where this technique is employed extensively to resort to temperature controlled rooms. However, where it is used less frequently such measures may be inconvenient.

To achieve similar control, we have found that a suitably sized box, in our own experience accommodating three tanks of usual size, may be maintained at an even temperature over long periods, by simply using a hair drier (fitted with the usual heating coil) in series with a mercury contact thermometer. The general arrangement is illustrated in Fig. 1.

The provision of a collapsible side finishing flush with the bottom of the box, allows for ease of removal of a tank and for the side to be used as a small bench if required.

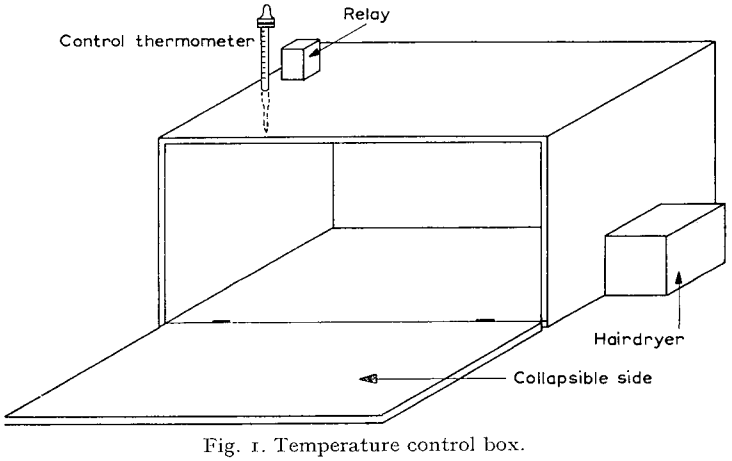


Fig. 1. Temperature control box.

This system has the advantage that as it is required heat is dissipated through the container by the blower. It is clear that the work required of the drier can be reduced by insulation and as a further contribution in this direction we have standardised on two temperatures, 20° and 25°, according to ambient temperature, with preference for the lower setting where possible.

Department of Applied Organic Chemistry,
University of New South Wales,
Kensington, N.S.W. (Australia)

P. H. BRADY
E. R. COLE

Received August 19th, 1963

Chromatography and photographic detection in ultraviolet light of 6-azauracil and its derivatives

For the purpose of analytical control of the biochemical transformation of 6-azauracil (3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine) to its riboside by *E. coli*, a rapid method was needed which could indicate the progress of the biotransformation. In order to be able to control the process (possible duration of a typical run is 8–10 h) the analysis should not take more than 1 h. A rapid qualitative chromatographic method on circular paper was found to meet this requirement, using either visual or photographic detection in U.V. light. The latter might be also used for a more elaborate semiquantitative method by the ascending technique.

Chromatographic technique

Paper chromatographic separation of purine and pyrimidine bases, nucleosides and nucleotides, was studied by HANDSCHUMACHER AND WELCH[1]. Generally, these compounds are first adsorbed on active charcoal, eluted by ammonia in methanol and after concentration developed by ascending paper chromatography in a butanol–water system[2–4]. We have found in model experiments that the rate of adsorption on charcoal is different for 6-azauracil (AU) and for 6-azauridine (AUR). In samples with low AUR content this fact can lead to considerable errors (up to 20 %). Polarographic studies in the course of the biotransformation process show that AUR in the fermentation fluid is strongly bound to high-molecular compounds, but that such complexes can be broken up by deproteinisation agents or simply by boiling. In samples treated in this way the charcoal step is no longer necessary and the samples can be directly spotted and developed with reproducible R_F values.

After extensive experimentation with several developing systems we have chosen the mixture *n*-butanol–acetic acid–water (12:1.5:5) which gives good resolution of the reaction components in a short time (R_F AU = 0.66, R_F AUR = 0.26).

Experimental

(1) *Rapid qualitative chromatographic separation of AU and AUR on circular paper.* Circular chromatographic paper Whatman No. 1 (diameter 15 cm) was spotted 1.5 cm

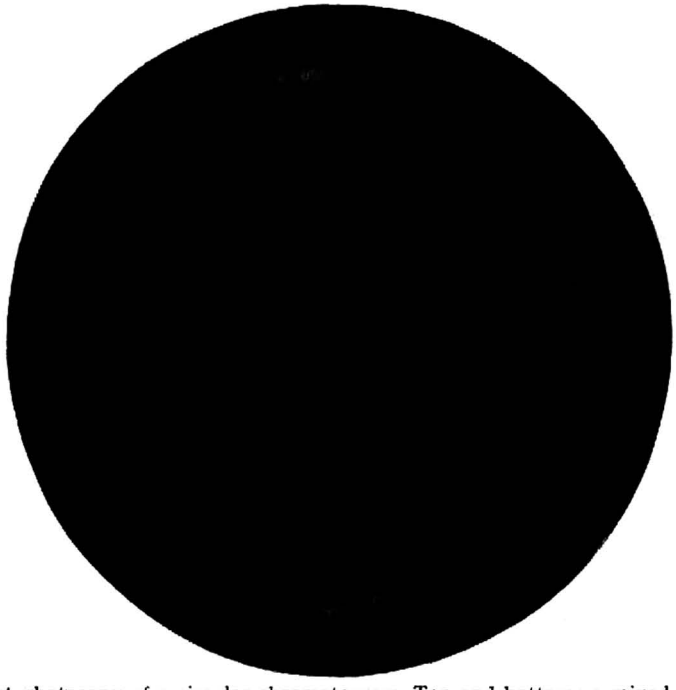


Fig. 1. Contact photocopy of a circular chromatogram. Top and bottom: a mixed standard; left and right: a sample of the fermentation medium. 5th hour of fermentation, AU is still untransformed.

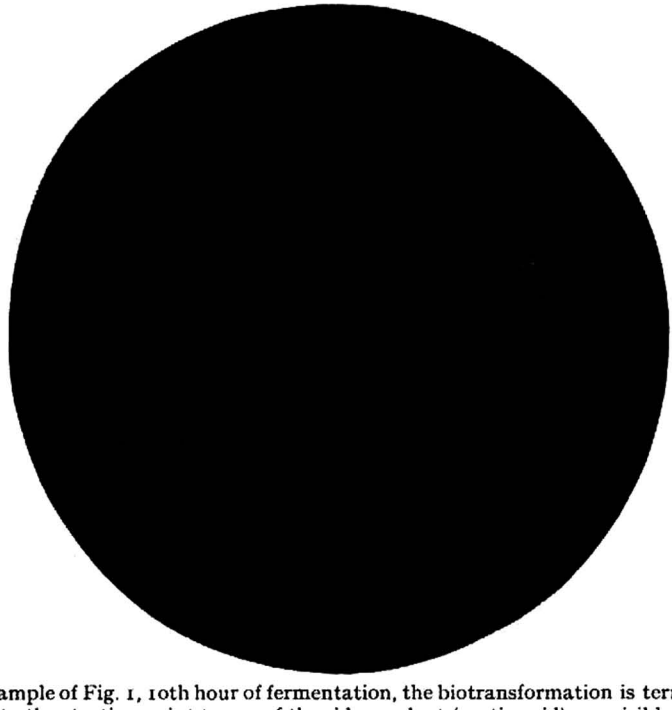


Fig. 2. Same sample of Fig. 1, 10th hour of fermentation, the biotransformation is terminated. Close to the starting point traces of the side-product (orotic acid) are visible.

from the centre alternatively with 10 μl aliquots of aqueous "mixed standard solution" (0.01 *M* AU and 0.01 *M* AUR) and with sample solutions. Fermentation fluid samples were warmed for 5 min in a test tube on a boiling water bath, cooled, centrifuged and the clear supernatant was directly spotted. The spots were dried in a current of hot air and the paper was positioned horizontally between two lids of Petri dishes (15 cm diameter). The developing solution was introduced to the centre of the paper by means of a paper wick, dipping in a small vessel containing the mixture. The inside of the Petri dishes was saturated with the developing mixture at least 4 h before starting the development. When the front of the solution was near to the circumference of the dish, the paper was taken out, dried and the spots were either directly observed in U.V. light (Hanau Mineralite), or the chromatograms were superimposed on photographic paper and prints were made by exposing them to a U.V. light source. This latter variant is described in detail below. In either case, the results are known within 1 h from starting the development of the chromatogram, which allows the biologist to follow the gradual conversion of AU to AUR in the course of the microbiological transformation (Figs. 1 and 2).

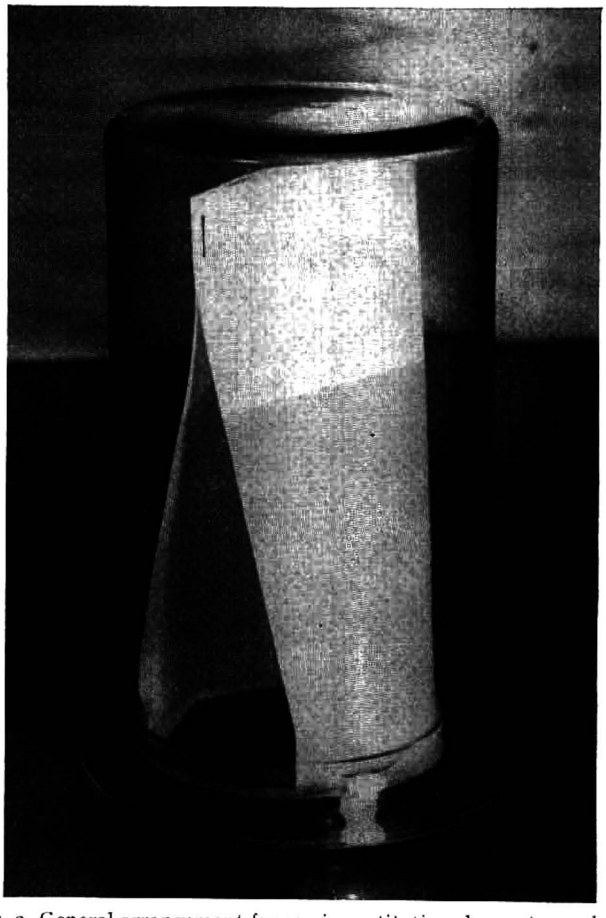


Fig. 3. General arrangement for semiquantitative chromatography.

(2) *Semiquantitative separation of AU and AUR by ascending paper chromatography*. For semiquantitative determination of AU and AUR in mixtures the ascending technique was used on 21 × 19 cm Whatman No. 1 paper sheets, folded in the shape of a cylinder. These sheets were placed in small Petri dishes, containing the developing mixtures (same as above), the whole assembly was put in an all-glass chamber (Fig. 3). Sample and "mixed standard solution" were applied in the same concentration as for the circular technique; the spottings were made in volumes of 20 μl 3 cm from the lower margin of the paper; the development took 3–4 h. After drying, photographic prints of each sheet were made and the prints were compared with a standard print, made from a chromatogram, to which a series of mixed standards was applied in molar percentual ratios AU to AUR of 100:0,90:10 ...10: 90, 0:100. In this way the ratio of AU to AUR in an unknown mixture can be determined with 5 % accuracy.

The method can be also applied for the separation of AUR derivatives. We show

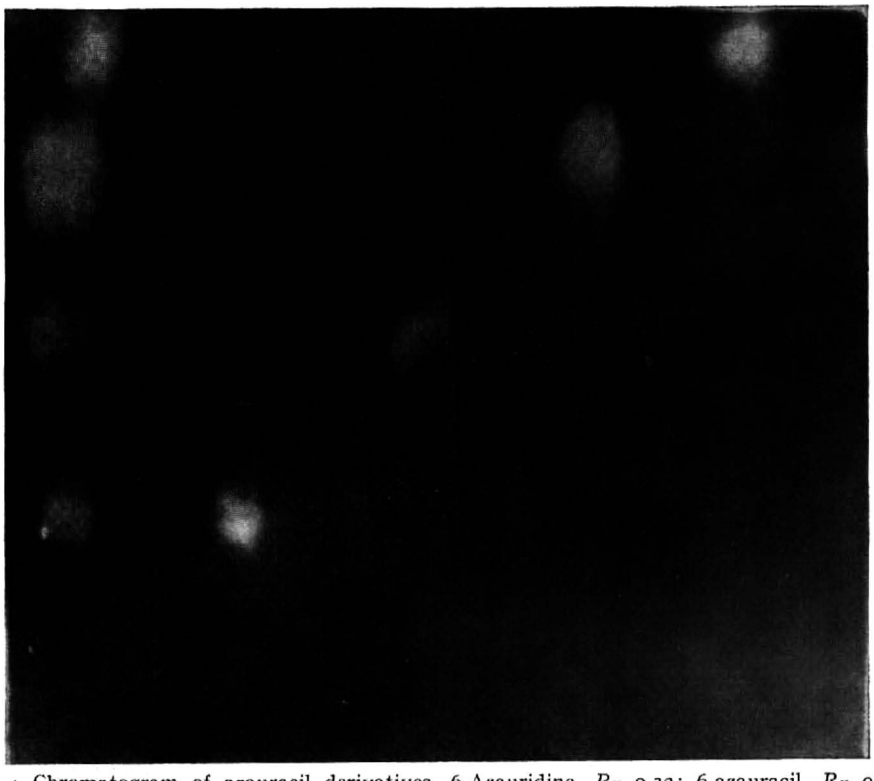


Fig. 4. Chromatogram of azauracil derivatives. 6-Azauridine, R_F 0.32; 6-azauracil, R_F 0.71; bromazauracil, R_F 0.88; 6-azauridine triacetate, R_F 0.96.

for example a photocopy of a mixture of 6-azauridine triacetate, bromazauracil, 6-azauracil, and 6-azauridine (Fig. 4).

Detection technique

Chemical reactions suitable for detection of purine and pyrimidine bases, nucleotides and nucleosides have been described by several authors[5–8]. Some reactions for the

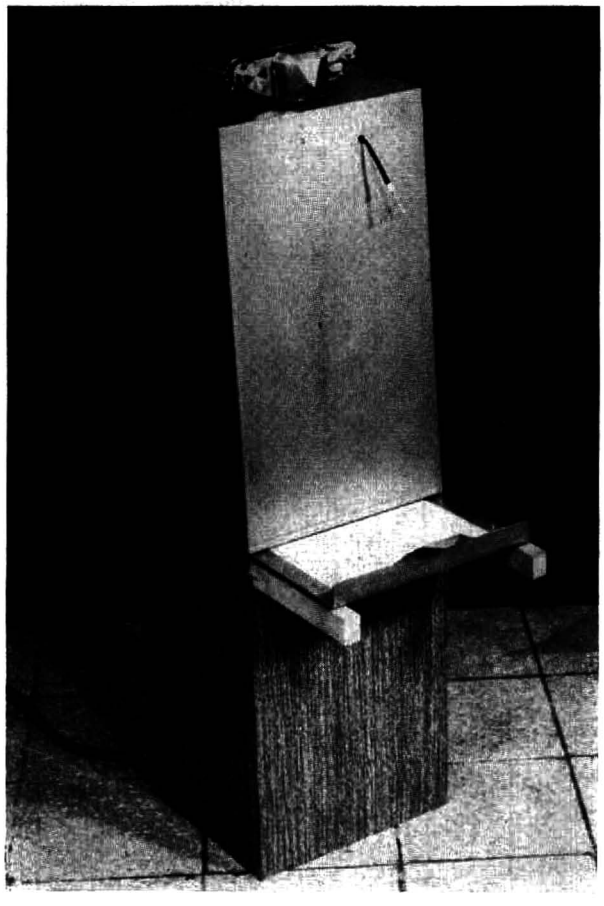


Fig. 5. Apparatus for detection by photography of the chromatograms in U.V. light. Distances: camera–chromatogram, 45 cm; chromatogram–U.V. lamp, 35 cm.

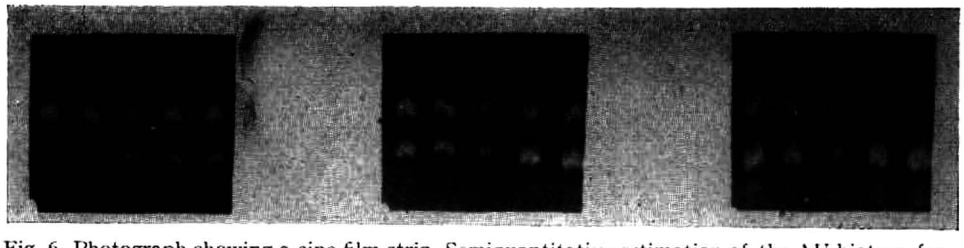


Fig. 6. Photograph showing a cine film strip. Semiquantitative estimation of the AU biotransformation process (0,3,5,7,9 and 10 h, "mixed standard solution" in the middle of each sheet).

phosphorus or sugar compounds may also be used[9], but chemical detection has several drawbacks. It is more laborious, less sensitive and it alters the chemical character of the detected substances which then cannot be used for other tests. The detection with U.V. light is therefore preferable. Low pressure mercury arc-lamps (*e.g.* Hanau Mineralite) are suitable for this purpose as long as they emit sufficient light in the wavelength range near 260 mμ.

During our work we tested several light sources and found that almost all low-pressure mercury arc-lamps yield adequate U.V. light quantities in the region of 260 mμ within a few seconds following ignition. This short time lapse is not long enough for visual perception and marking of the spots but it proves adequate for the exposure of the photographic paper. This revelation was utilized for the development of the contact photocopy detection method.

Under nonactinic light the dried chromatograms were superimposed on a sensitive photographic paper, weighted with a metal frame and exposed to the light of a mercury arc-lamp (Fig. 5). The exposure time and focal distance of the lamp must be checked and determined according to the sensitivity of the paper and the intensity of the lamp. If normal photographic papers for enlargement and a 250 W lamp are used, the focal distance is 2 m and the exposure time 4 sec. The exposed papers were developed by means of a hard contrast developer.

More convenient still, and less time consuming is the detection by photographing on a cine film (Fig. 6). The prerequisite necessary for the development of this method was again a suitable light source. Several available U.V. lamps were tested using the spectrophotometer Unicam SP 700 and the one-beam technique. The lamp under test served as light source and the intensities of the lines were measured using several split-widths for comparison. The germicidal lamp TUW 30 was found to be best for our work. The photographic apparatus used was Exacta Varex, cine film 17/10 din, exposure time 2 sec. The required quartz lens-set was made in the Research Institute for Monocrystals, Turnov.

The filing of chromatographic documents thus obtained is very convenient. The size and intensity of the zones can best be read by means of a slide-projector on a screen.

*Research Institute of Antibiotics**,
Roztoky u Prahy (Czechoslovakia)

V. BULANT
O. HORSKÝ
M. URX

[1] R. E. HANDSCHUMACHER AND A. D. WELCH, *Cancer Res.*, 16 (1956) 965.
[2] J. ŠKODA, V. F. HESS AND F. ŠORM, *Chem. Listy*, 51 (1957) 1194.
[3] J. ŠKODA, AND F. ŠORM, *Chem. Listy*, 50 (1956) 1165.
[4] J. ŠKODA, V. F. HESS AND F. ŠORM, *Experientia*, 13 (1957) 150.
[5] E. CHARGAFF, E. VISCHER, R. DONIGER, CH. GREEN AND F. MISANI, *J. Biol. Chem.*, 177 (1949) 405.
[6] E. CHARGAFF, B. MAGASANIK, E. VISCHER, CH. GREEN, R. DONIGER AND D. ELSON, *J. Biol. Chem.*, 186 (1950) 51.
[7] R. M. REGUERA AND I. ASIMOV, *J. Am. Chem. Soc.*, 72 (1950) 5781.
[8] B. MAGASANIK, E. VISCHER, R. DONIGER, D. ELSON AND E. CHARGAFF, *J. Biol. Chem.*, 186(1950) 37.
[9] C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.

First received April 10th, 1963
Modified July 16th, 1963

* Director Dr. Ing. M. HEROLD, D. Sc.

Chromato-stack, a simple technique for improving the efficiency of thin-layer chromatography

In a recent paper[1] the author has described a new system for thin-layer chromatography using discarded photographic plates as glass supports. The plates are arranged in long rows on a desk and a simple spreading device is used to coat several plates at once.

The 9 × 12 cm photographic plates may take 5 or 6 samples, whereas the 12 × 16 cm plates correspond to ordinary chromatographic paper sheets and take 10–12 samples. Ordinary developing tanks hold only 2 plates at a time but the Chromato-stack technique described here makes it possible to develop 8–12 plates simultaneously. The technique is very simple, though efficient. The only equipment necessary consists of ordinary rubber bands and small plastic pellets, such as are obtained when punching 5 mm holes in 2–3 mm thick polythene sheets.

Figs. 1–4 show the principle. After spotting the various extracts or solutions, the plates are put on the desk. On each corner of a plate a plastic pellet is placed (Fig. 1). A new plate is put on the top of the first one, four new pellets are placed in the corners again (Fig. 2) and in this way the stack is successively built up. Finally, a clear plate is put on the top, and the stack is fastened together with rubber bands (Fig. 3). Thereafter the stack may be put into a suitable glass (or polythene) tank for developing (Fig. 4).

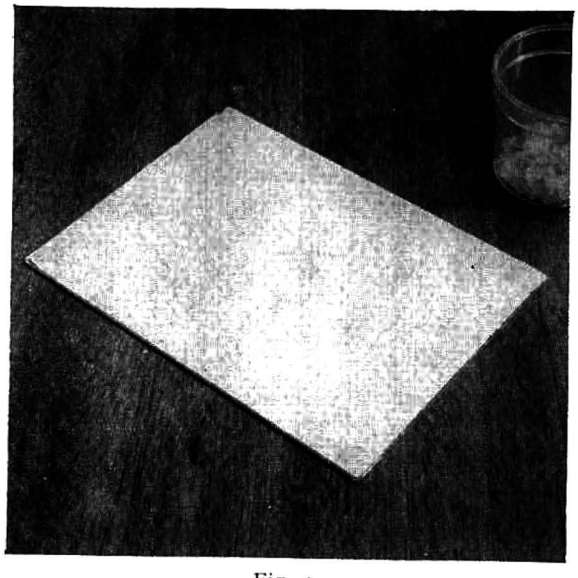


Fig. 1.

Simultaneous development of 60–120 samples (usually in less than one hour) is not the only advantage of the Chromato-stack technique. The narrow air spaces between the plates promote equilibrium of the vapour pressure, which is very important in the case of many developing solvents.

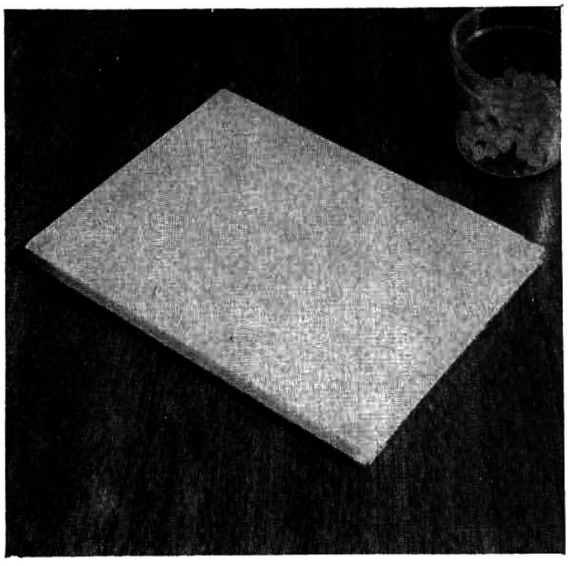


Fig. 2.

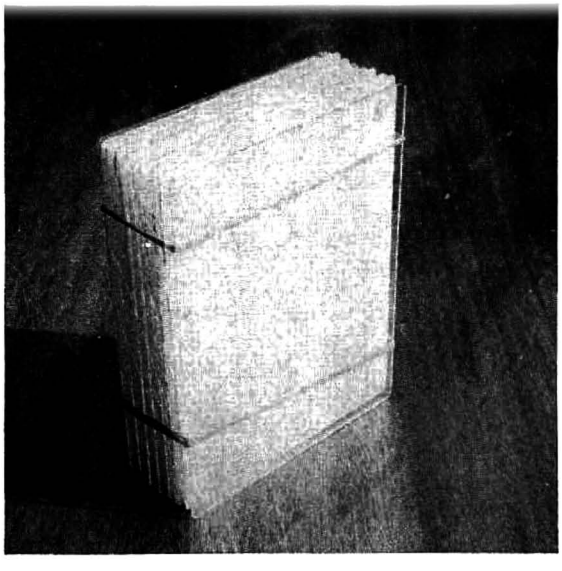


Fig. 3.

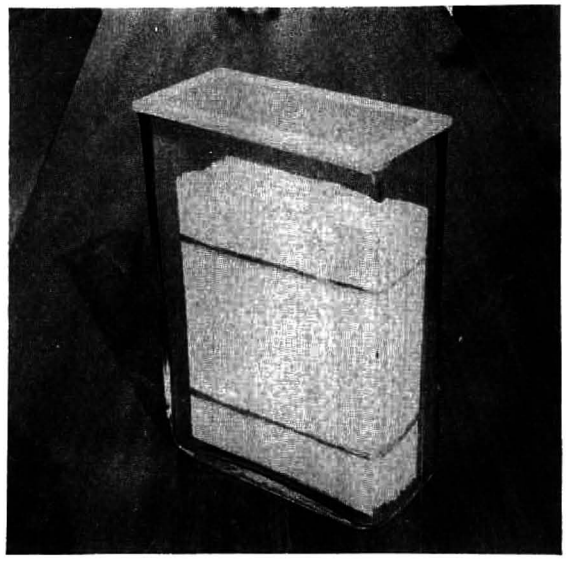


Fig. 4.

If the stacks become very large and heavy, it may be advisable to have two wire hooks for lifting them into and out of the tank. It is evident that the technique is also suitable for the simultaneous development of two-dimensional plates.

Balsgård Fruit Breeding Institute,
Fjälkestad (Sweden)

NILS NYBOM

[1] N. NYBOM, *Nature*, 198 (1963) 1229.

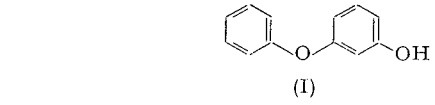
Received July 17th, 1963

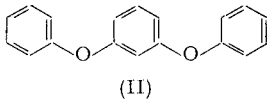
Separation of polyphenyl ethers by thin-layer chromatography

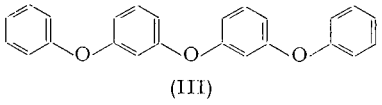
A multiple development technique

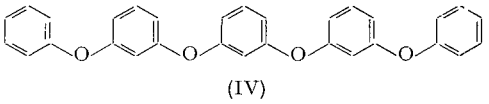
During an investigation of the mechanism of the oxidation at high temperatures of polyphenyl ethers, a method was needed for separating the oxidation products. Among the model compounds chosen for study were the following: *m*-phenoxyphenol (I), *m*-diphenoxybenzene (II), bis-(*m*-phenoxyphenyl) ether (III), and *m*-bis-(*m*-phenoxyphenoxy)-benzene (IV).

Initial attempts at separating a mixture of the above compounds by column chromatography on silica gel with various solvents were unsuccessful, so thin-layer chromatography was attempted in hopes of finding suitable conditions for the separation. It was found that a single elution and development of the above compounds with the most suitable solvent system did not give satisfactory separation but that multiple


(I)


(II)


(III)


(IV)

development of thin-layer chromatograms provided efficient separation of a mixture of I, II, III, and IV.

The *m*-phenoxyphenol and bis-(*m*-phenoxyphenyl) ether were commercially available. The *m*-diphenoxybenzene and *m*-bis-(*m*-phenoxyphenoxy)-benzene were kindly supplied by Dr. GLENN R. WILSON of this laboratory and were distilled before use. The chloroform, cyclohexane, and benzene used were reagent-grade and were used without further purification. A 1 % (by wt.) solution in chloroform of each of the above compounds was prepared by dissolving 0.377 g of the compound in exactly 25.00 ml of chloroform. A synthetic mixture of I, II, III, and IV was prepared by mixing 5 ml of each of the 1 % chloroform solutions.

Thin-layer chromatograms were prepared in the usual manner. They consisted of a 250-μ layer of Silica Gel G on a 20 × 20 cm glass plate applied with a fixed thickness spreader (Research Specialties Company, Richmond, Calif.).

Five separate plates were carefully spotted with a Lang-Levy micropipette (10 λ) with each of the above solutions including the synthetic mixture. This permitted easy comparison of the distance traveled by the pure compounds with the distance traveled by the compounds in a mixture and the identification of the compound in the mixture.

The chromatoplates were activated in an oven at 105–110° for 0.5 h before each development except in the one case noted below. It was found by application of the "Micro Circular Technique"[1], that the best development solution was 5 % benzene – 95 % cyclohexane. The development chambers were standard size and the larger walls were covered with Whatman No. 1 filter paper saturated with the benzene–cyclohexane solution to minimize "edge effects" and the "fringe phenomenon".

The two methods described below were used to determine the effects of multiple development on the separation of the polyphenyl compounds.

Method A

Four plates, activated and spotted identically were developed simultaneously. After drying in air, one plate was sprayed with a fluorescent indicator (Aldrich Chemical Co., Fluorescent indicator for hydrocarbons) and viewed under U.V. light. The distances traveled for each pure compound and for each compound in the mixture were recorded. The remaining three plates were activated at 110° for 0.5 h and were subjected to another development. Again one plate was used to determine the distances traveled. The third plate was *not* reactivated before an additional development to determine whether reactivation was necessary to significantly increase the distances traveled. The fourth plate was activated before a third and fourth development.

Method B

A single chromatoplate, after activation, was spotted with the above solutions and developed in the same manner. However, instead of spraying with fluorescent indicator, the location of each of the compounds was determined by treatment with iodine vapor. Once the spots were located and distances traveled tabulated, the iodine was allowed to evaporate from the chromatoplate. After the iodine had completely evaporated, the plate was reactivated at 110° for 0.5 h and redeveloped. The procedure was repeated three times and the distance traveled by each spot was recorded after each iodine treatment.

In all developments, the solution was allowed to travel from one end of the chromatoplate to a line drawn across the plate at a distance of 10 cm from the position of the spots. Since the distance between the spots and the line to which the solvent travels decreases with each development, one cannot use "R_F" values to refer to the distances traveled by the spots except for the very first development. The easiest way to record data in multiple developments is simply to note the total distance the spot has traveled from its original position. These values for the pure compounds I, II, III, and IV from method A and B are recorded in Table I.

TABLE I

DISTANCES TRAVELED ON MULTIPLE DEVELOPMENT (cm × 10^{-1})

Compound	*Development number*							
	Method A				*Method B*			
	1	*2*	*3**	*4*	*1*	*2*	*3*	*4*
I	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
II	0.25	0.47	0.50	0.65	0.25	0.45	0.60	0.68
III	0.17	0.33	0.36	0.46	0.15	0.30	0.40	0.50
IV	0.10	0.18	0.22	0.30	0.10	0.19	0.26	0.32

* Chromatoplate not activated before final development.

The values of the distance traveled for the compounds in the synthetic mixture are recorded in Table II.

A slight impurity was found in "pure samples" of I and II after the four development. It had traveled about 0.08 cm and therefore was neither of the higher phenyl ethers being studied.

The correlation between the two methods is good considering the experimental error. The multiple development technique significantly increases the separation for the compounds separated. While no system could be found that cleanly separated these compounds with a single development, two developments were sufficient for clean separation. It seems necessary to activate the plates after each development since development 3, Method A, showed very little change in the distance traveled while a similar activated plate, development 3, Method B, showed significant changes.

It should be mentioned that the polyphenyl ethers are base fluids for high temperature lubricants and because of their high thermal stability there is little

TABLE II

DISTANCES TRAVELED ON MULTIPLE DEVELOPMENT OF SYNTHETIC MIXTURE (cm × 10^{-1})
The braces denote overlap of spots; a is about 20% overlap, b is about 50% overlap

Compound		Development number							
		Method A				Method B			
		1	2	3*	4	1	2	3	4
I		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
II	a	0.23	0.48	0.48	0.64	0.24	0.43	0.57	0.65
III	a	0.14	0.29	0.30	0.43	0.20	0.27	0.38	0.45
IV	b	0.09	0.16	0.17	0.30	0.10	0.13	0.22	0.29

* Chromatoplate not activated before final development.

danger of reaction or rearrangement on heating to 110° in the presence of silica gel. Other systems may not be so obliging.

These results have been used to obtain an efficient separation of the above compounds and similar polyphenyl ethers in column chromatography.

Monsanto Research Corporation,
Boston Laboratories, Everett, Mass. (U.S.A.)

RICHARD H. NEALEY

[1] E. STAHL, *Chemiker Ztg.*, 82 (1958) 323.

Received August 19th, 1963

Die Anwendung der Dünnschichtchromatographie zur Trennung des 2,5,7,8-Tetramethyl-2-(β-carboxyäthyl)-6-hydroxychromans und des Tocopheronolactons von α-Tocopherylchinon und einigen Tocopherolen

Untersuchungen von SIMON und Mitarbeitern[1] über den Wirkungsmechanismus des α-Tocopherols zeigten, dass das Tocopheronolacton [Lacton des 2-(3-Hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzochinons] ein Umwandlungsprodukt des α-Tocopherols im tierischen Organismus darstellt. Von MARTIUS UND FÜRER[2] wird das 2,5,7,8-Tetramethyl-2-(β-carboxyäthyl)-6-hydroxychroman als eine sehr wahrscheinliche Abbaustufe des α-Tocopherols durch β-Oxydation angesehen. Beide genannten Verbindungen sind von WEICHET und Mitarbeitern[3] bereits synthetisch hergestellt worden*. Wegen ihrer grossen Bedeutung sollte geprüft werden, welche Möglichkeiten bestehen, 2,5,7,8-Tetramethyl-2-(β-carboxyäthyl)-6-hydroxychroman, Tocopheronolacton, α-, γ-, δ-Tocopherol und α-Tocopherylchinon mit Hilfe der Dünnschichtchromatographie voneinander zu trennen. Die Ergebnisse sind in Tabelle I zusammenge-

* Herrn Dr. WEICHET vom Institut für Pharmazie und Biochemie, Prag, danke ich herzlich für die Überlassung von Substanzproben.

TABELLE I

R_F-WERTE DER UNTERSUCHTEN VERBINDUNGEN

A_1: Zinkcarbonat–Aluminiumoxyd (1:3).
A_2: Zinkcarbonat–Silicagel (1:1).
S_1: Aceton–paraffingesättigtes Wasser (9:1).
S_2: Chloroform.
S_3: Acetonitril–Wasser (9:1).
S_4: Benzol–Chloroform (1:1).

Verbindungen	R_F			
	A_1		A_2	
	S_1	S_2	S_3	S_4
2,5,7,8-Tetramethyl-2-(β-carboxyäthyl)-6-hydroxychroman	0.34	0.00	0.30	0.00
Tocopheronolacton	1.00	0.59	1.00	0.61
α-Tocopherylchinon	1.00	0.58	1.00	0.75
α-Tocopherol	1.00	0.72	1.00	0.83
γ-Tocopherol	1.00	0.42	1.00	0.80
δ-Tocopherol	1.00	0.31	1.00	0.67

fasst widergegeben. Eine gute Trennung des 2,5,7,8-Tetramethyl-2-(β-carboxyäthyl)-6-hydroxychromans von allen aufgeführten Verbindungen erreicht man mit dem Adsorbens Zinkcarbonat–Aluminiumoxyd (5 g Zinkcarbonat, 15 g Aluminiumoxyd nach Brockmann, 20 ml Wasser, 2 ml Wasserglas) und dem Lösungsmittelsystem Aceton–paraffingesättigtes Wasser (9:1)[4] oder mit Zinkcarbonat–Silicagel (10 g Zinkcarbonat, 10 g Silicagel, 40 ml Wasser, 2 ml Wasserglas) und dem Laufmittel Acetonitril–Wasser (9:1)[5]. Das Tocopheronolacton lässt sich am besten in dem System Zinkcarbonat–Silicagel mit Benzol–Chloroform (1:1)[4] von den anderen Verbindungen mit Ausnahme des δ-Tocopherols abtrennen. Eine ausreichende Trennwirkung der aufgeführten Tocopherole wird mit Zinkcarbonat–Aluminiumoxyd und Chloroform als Laufmittel erzielt; dabei stören das Tocopheronolacton, das 2,5,7,8-Tetramethyl-2-(β-carboxyäthyl)-6-hydroxychroman und α-Tocopherylchinon nicht.

Die Versuche wurden mit Glasplatten 12 × 18 cm durchgeführt, die aufgebrachte Adsorbensmischung 25 min bei 110° aktiviert. Bei einer Steighöhe von 15 cm und einer Schichtstärke von 0.50 mm beträgt die Laufzeit mit Benzol–Chloroform (1:1) 60 min, in allen anderen Systemen 30–35 min. Als Sprühreagenz zum Nachweis aller untersuchten Verbindungen eignet sich eine 0.2%ige äthanolische Rhodamin-B-Lösung[4].

Institut für Ernährung der Deutschen Akademie der Wissenschaften zu Berlin, Potsdam-Rehbrücke (Deutschland)

H. SCHMANDKE

[1] E. J. SIMON, A. EISENGART, L. SUNDHEIM UND A. T. MILHORAT, *J. Biol. Chem.*, 221 (1956) 807.
[2] C. MARTIUS UND E. FÜRER, *Biochem. Z.*, 336 (1963) 474.
[3] J. WEICHET, L. BLÁHA UND B. KAKÁČ, *Collection Czech. Chem. Commun.*, 24 (1959) 1689.
[4] H. WAGNER UND B. DENGLER, *Biochem. Z.*, 336 (1962) 380.
[5] H. H. DRAPER, A. SAARI CSALLANY UND S. N. SHAH, *Biochim. Biophys. Acta*, 59 (1962) 527.

Eingegangen am 6. August 1963

Eine neue spezifische Farbreaktion zum papier- und dünnschichtchromatographischen Nachweis des Adenins

Die papier- und dünnschichtchromatografischen Farbreaktionen der Purin- und Pyrimidinbasen, bzw. ihrer Derivate, sind dadurch charakterisiert, dass sie alle die gleichfarbige oder nahe gleichfarbige Farbreaktion geben, so ist die Identifizierung manchmal schwer[1–4]. In dieser Mitteilung behandeln wir die spezifische Farbreaktion des Adenins, welche auf die Wirkung des entsprechenden Dragendorff-Reagenzes und nach der Entwicklung auf die Wirkung der darauffolgenden verdünnt schwefelsauren, bzw. perchlorsauren Besprühung auftritt.

*Reagenzen**

(a) Dragendorff-Reagenz. 7.0 g Natriumjodid und 2.6 g basisches Wismutcarbonat wird 20 Min lang in 20 ml Eisessig, in einer Destillierkolben, welcher mit einem Rückflusskühler versehen ist, gekocht (die Lösung ist dunkelrot!). Nachher wird 80 ml Äthylacetat dazugegeben, zusammengeschüttelt und für 2 Tage in den Kühlschrank gestellt. Von den ausgeschiedenen Natriumacetatkristallen wird die Lösung nachher kalt abfiltriert und dem Filtrat wird 0.5 ml dest. Wasser dazugegeben (ca. 100 ml Stammlösung).

Verdünnung zum Papier: 10 ml Stammlösung, 25 ml Eisessig, 60 ml Äthylacetat; zur Dünnschicht: 10 ml Stammlösung, 100 ml Eisessig, 240 ml Äthylacetat.

(b) Schwefelsäure. Papier: $N/20$ H_2SO_4; Dünnschicht: 10 %-ige H_2SO_4.

(c) Perchlorsäure. Papier: 0.14 %-ige $HClO_4$; Dünnschicht: 10 %-ige $HClO_4$.

Lösungsmittelgemische

Zur Papierchromatographie:

(a) Destilliertes Wasser[5],

(b) *n*-Butanol–Eisessig–Wasser[6] (4:1:5).

Zur Dünnschichtchromatographie:

(a) Destilliertes Wasser[7],

(b) Chloroform–Methanol–Wasser (65:25:4).

Papiere, Adsorbenten

Schleicher und Schüll 2043b, Kieselgel G, Szialgel 47** (ein siliciumoxyd- und aluminiumoxydhältiger Adsorbent).

Besprechung der Ergebnisse

VÁGUJFALVI[8] fand bei dem Nachweis der Alkaloiden, dass nach der sogenannten nichtwässrigen Dragendorff-Reagenz-Entwicklung, und der darauffolgenden $N/20$ schwefelsauren Besprühung, die Empfindlichkeit des Nachweises bei den meisten Alkaloiden sich steigerte. Diese Erscheinung haben wir bei den Purin- und Pyrimidinbasen, sowie bei ihren Derivaten ausprobiert, und wir fanden, dass mit dem Dragendorff-Reagenz nur das Adenin eine blasse zitronengelbe Reaktion gibt, aber wenn man das Papier nach der Trocknung in $N/20$ H_2SO_4 oder in 0.14 %-ige $HClO_4$ Lösung ein-

* Bei den Untersuchungen war mich E. SIMONFAI behilflich.

** Unter Herstellung bei der Firma Reanal, Budapest.

taucht, liefert das Adenin eine intensiv blutrote Reaktion, welche einen grösseren Platz auf dem Papier einnimmt als die originale zitronengelbe Reaktion (Fig. 1). Das Hypoxantin gibt die Reaktion nur zum Teil, mit blasser, orangegelber Farbe. Diese Erscheinung ist auch auf dem Dünnschicht wahrnehmbar.

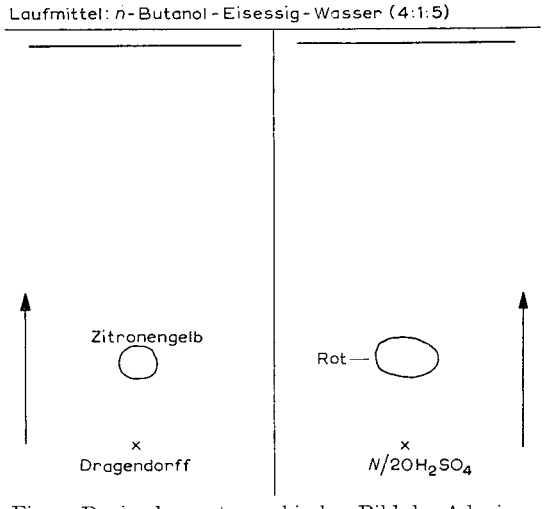


Fig. 1. Papierchromatographisches Bild des Adenins.

Die anderen Nucleinbasen und Derivate reagierten auf die Nachbehandlung nur mit blassgelber, nicht charakteristischer Farbe, oder gar nicht. Das AMP (Adeninmonophosphat) ergibt auf die Wirkung der Nachbehandlung eine blasse orangegelbe Farbe. Das ATP (Adenintriphosphat) ergibt eine ganz blassgelbe Reaktion.

Die verdünnte perchlorsäurige Nachbehandlung ergab einen der schwefelsaurigen Entwicklung ähnlichen Ergebnis.

Die Grenze des Nachweises war 1 γ.

Dank

Der Firma Zellstoffabrik Waldhof danke ich an dieser Stelle für die Testsubstanzen.

Institut für Heilpflanzenforschung, Budapest (Ungarn) E. TYIHÁK

[1] E. CHARGAFF, E. VISHER, G. DONIGER, G. GREEN UND F. MISANI, *J. Biol. Chem.*, 177 (1949) 405.
[2] H. MICHL, *Naturwiss.*, 40 (1953) 390.
[3] R. M. REGUERA UND I. ASIMOV, *J. Am. Chem. Soc.*, 72 (1950) 5781.
[4] T. WOOD, *Nature*, 176 (1955) 175.
[5] C. TAMM, H. S. SHAPIRO, R. LIPSHITZ UND E. CHARGAFF, *J. Biol. Chem.*, 203 (1953) 673.
[6] P. BOULANGER UND J. MONTREUIL, *Bull. Soc. Chim. Biol.*, 33 (1951) 784, 791.
[7] K. RANDERATH, *Angew. Chem.*, 73 (1961) 674.
[8] D. VÁGUJFALVI, *Planta Med.*, 8 (1960) 34.

Eingegangen den 20. August 1963

Thin-layer chromatography of erythromycins

Ascending paper chromatography has been used in the analysis of erythromycins[1–3]. In our laboratory, however, it became desirable to develop a method of chromatographic analysis which would be more rapid, sensitive, and less subject to temperature variation. As a result, a thin-layer chromatographic procedure for separating erythromycins on silica gel plates was developed.

A method for the thin-layer chromatographic separation of cardiac glycosides using methylene chloride–methanol–formamide (80:19:1) was described by STAHL[4]. Since the erythromycin molecule contains sugar residues similar to the cardiac glycosides, attempts were made to use the above solvent system for the thin-layer chromatographic separation of erythromycins. Satisfactory results were obtained after the solvent system was modified to include benzene and variable amounts of formamide.

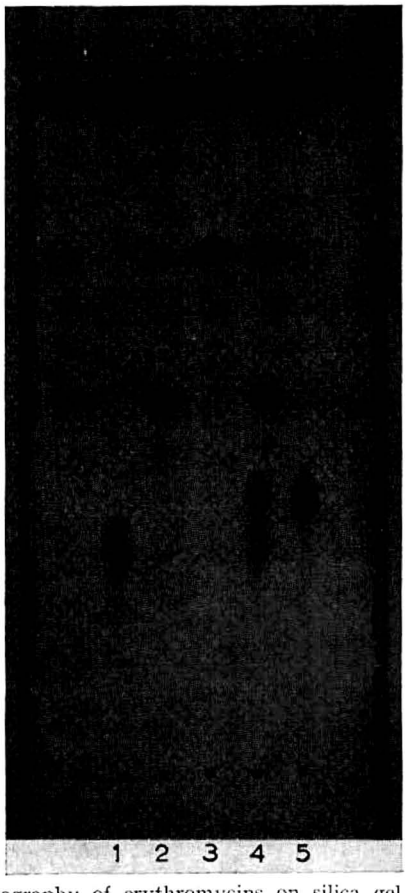


Fig. 1. Thin-layer chromatography of erythromycins on silica gel G plates. Solvent system: methylene chloride–methanol–benzene–formamide (80:20:20:3); (relative humidity 26%). (1) erythromycin A (5 γ); (2) 4″-acetylerythromycin A* (5 γ); (3) 2′,4″-diacetylerythromycin A* (5 γ); (4) mixture of 1, 2, 3 and 5 (10 γ); (5) erythromycin A "Hemiketal"[2] (5 γ). Time of development 30 min; spot detection with 50% aqueous H_2SO_4 spray and charring.

* Prepared by Dr. P. H. JONES, Abbott Laboratories; procedure to be published.

Glass plates (5 × 20 cm) were coated and activated according to the method of STAHL[5], using silica gel G (Merck) as the adsorbent layer. The solvent system used for the development of the plates consisted of methylene chloride–methanol–benzene–formamide (80:20:20:2–5 v/v/v/v). The amount of formamide in the system was varied according to the humidity conditions present in the laboratory. At a relative humidity of 20%, 5 volumes of formamide in the solvent system gave clear separation of erythromycin A and B. Higher relative humidity conditions (30–40 %) required 3 or 2 volumes of formamide for separation.

The time of development varied from 30–40 min. Chromatographic separation was completed when the solvent front advanced to 10 cm from the origin.

Detection of the spots was carried out by spraying the plate with 10 % phosphomolybdic acid in ethanol. Upon heating on a hot plate, blue spots appeared on a yellow background. Spraying the plate with 50 % aqueous H_2SO_4 and heating on a hot plate charred the erythromycins. Although the phosphomolybdic acid spray is

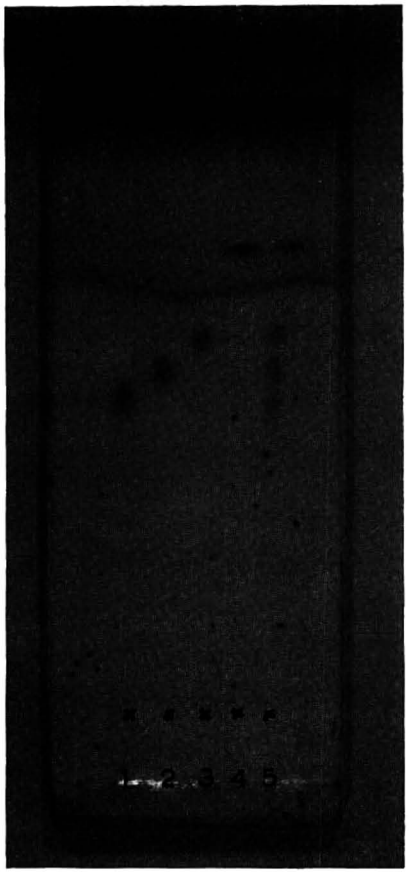


Fig. 2. Thin-layer chromatography of erythromycins on silica gel G plates. Solvent system: methylene chloride–methanol–benzene–formamide (80:20:20:4); (relative humidity 25 %). (1) erythromycin A (2 γ); (2) erythromycin B (2 γ); (3) anhydroerythromycin[6] (2 γ); (4) 2'-acetylerythromycin[7] (2 γ); (5) mixture of 1, 2, 3 and 4 (4 γ). Time of development 40 min: spot detection with 50 % aqueous H_2SO_4 spray and charring.

more sensitive, the spots tend to fade rapidly one to two hours after spraying. Charring with H_2SO_4 is preferable if the spots should remain visible for documentation.

Erythromycin A and B, erythromycin A "Hemiketal"[2], anhydroerythromycin[6], and several erythromycin acetates were separated using the methylene chloride–methanol–benzene–formamide solvent system (Figs. 1 and 2).

Thin-layer chromatography on silica gel G plates using the methylene chloride–methanol–benzene–formamide system has proven to be an efficient and sensitive method for the separation of erythromycins. This procedure is preferable to paper chromatography since the time of analysis is greatly decreased.

Acknowledgement

The author wishes to thank Dr. P. H. JONES for supplying the erythromycin samples and giving guidance and encouragement.

Organic Chemistry Department, Research Division,
Abbott Laboratories, North Chicago, Ill. (U.S.A.) TAIMI T. ANDERSON

[1] M. PIECHOWSKA AND B. OSTROWSKA-KRYSIAK, *Med. Doswiadczalna Mikrobiol.*, 13 (1961) 167.
[2] V. C. STEPHENS AND J. W. CONINE, *Antibiot. Ann.*, (1958–1959) 346.
[3] V. ŠEVČÍK, M. PODOJIL AND A. VRTIŠKOVÁ, *Folia Biol. (Prague)*, 3 (1957) 218.
[4] E. STAHL AND U. KALTENBACH, *J. Chromatog.*, 5 (1961) 458.
[5] E. STAHL, *Chemiker Ztg.*, 82 (1958) 323.
[6] P. F. WILEY, K. GERZON, E. H. FLYNN, M. V. SIGAL, O. WEAVER, U. C. QUARCK, R. R. CHAUVETTE AND R. MONAHAN, *J. Am. Chem. Soc.*, 79 (1957) 6062.
[7] V. C. STEPHENS, *Union of South Afrika Patent* 2616/58, July 11, 1958.

Received August 5th, 1963

J. Chromatog., 14 (1964) 127–129

Bioautography of antibiotic spread-layer chromatograms

Thin-layer chromatography (TLC) of antibiotics is receiving increasing attention[1–6]. In comparison with paper chromatography, TLC has greater resolving power, is the more rapid of the two techniques and can be more easily scaled up to preparative scale. A necessary adjunct to any chromatographic technique is a means of detection; with TLC, U.V. absorption, U.V. fluorescence, color producing reagents, charring with mineral acids and heat, and bioautography are methods in use. For antibiotics, bioautography is preferable and necessary because it is usually more sensitive than chemical methods and detects only biologically active components.

Several reports in the literature describe bioautography of antibiotics separated by TLC. NICOLAUS, CORONELLI AND BINAGHI[1] poured agar seeded with *Bacillus subtilis* over the developed chromatographic plate, while BICKEL *et al.*[3] and BRODASKY[5] pressed the chromatographic plate onto seeded agar plates. These investigators used adsorbents containing binder. In our studies, we have used the spread-layer technique[7–9], in which no binder is present in the adsorbent. Some advantages of this method are: alumina plates can be prepared and used within a matter of minutes, alumina of the desired activity can be used and the need for costly spreading devices

is eliminated. The purpose of this communication is to describe a means for the bioautography of such chromatograms. The applicability of this method to antibiotics with different biological activities will be demonstrated.

Experimental

Alumina (Woelm) and silica gel H (Merck) were spread on 3 × 8 in. glass plates by means of a glass rod. A double thickness of adhesive tape was wrapped about each end of the glass rod so that the tape rested on the edges of the glass plate when the rod was held in a horizontal position. The thickness of the tape wrapping determines the clearance between the glass rod and the surface of the plate, and hence the thickness of the adsorbent layer. The rod is drawn back and forth over the plate several times, removing any surface imperfections and excess adsorbent. The adsorbent layers used in our studies were approximately 0.5 mm thick. Alumina, of the required activity, was spread as a dry powder and silica gel H as an aqueous slurry prepared by the addition of 2–2.5 parts water. The alumina plates were ready for immediate use whereas the silica gel H plates were air dried at room temperature overnight before use. The antibiotics and chromatographic systems used were: thiostrepton — neutral alumina adjusted to activity IV, developed with 5 % methanol in chloroform; erythromycin, nystatin and polymyxin B sulfate — silica gel H developed with methanol.

For bioautography with bacteria, 200 ml of Base Agar and 100 ml Seed Agar (Baltimore Biological Laboratories, Baltimore, Maryland), each with added 0.5 % sodium chloride, were poured into 8 × 13 in. sterile Pyrex baking dishes covered with aluminum covers. The Seed Agar contained 1 ml of an aqueous 2 % (w/v) solution of 2,3,5-triphenyl-2H-tetrazolium chloride and 2.5 ml of a 24 h broth culture of the indicator organism. *Staphylococcus aureus* 209 P and *Escherichia coli* ATCC 10536 were grown in Penassay Broth (Difco Laboratories, Detroit, Michigan) and *Streptococcus lactis* (Squibb Culture Collection No. 1783) in Micro Inoculum Broth (Difco). For the yeast, *Saccharomyces cerevisiae* (Squibb Culture Collection No. 1600), the agar medium was of the following composition: tryptone, 5 g; malt extract, 3 g; glucose, 10 g; yeast extract, 3 g; agar, 15 g; distilled water, 1 l. This medium, lacking agar, was used to prepare the inoculum, which was 2.5 ml of a 24 h culture incorporated into the seed agar. No dye was added to this medium.

For bioautography, a 3 × 11 in. sheet of Whatman No. 1 paper, wetted by dipping through distilled water, was centered over a clean glass plate of the same size as the chromatographic plate. The filter paper tabs, extending beyond each end of the glass plate, were folded back over the glass plate and the assembly carefully placed on top of the chromatographic plate, resulting in a sandwich, with the filter paper and adsorbent layer between the glass plates. The filter paper tabs were folded back over the ends of the chromatographic plate, and the sandwich inverted so that the chromatographic plate was on top and the glass support plate on bottom. The latter was set aside, leaving the filter paper adhering to the glass chromatographic plate by means of the filter paper tabs. This assembly was carefully placed on the surface of the seeded agar.

Initially, our technique consisted of incubating the developed chromatographic plate on the agar surface seeded with *S. aureus* 209 P for 16 h at 37°. This method was unsuccessful because the organism did not grow under the chromatographic plate. The technique was modified by removing the chromatographic plate and filter paper

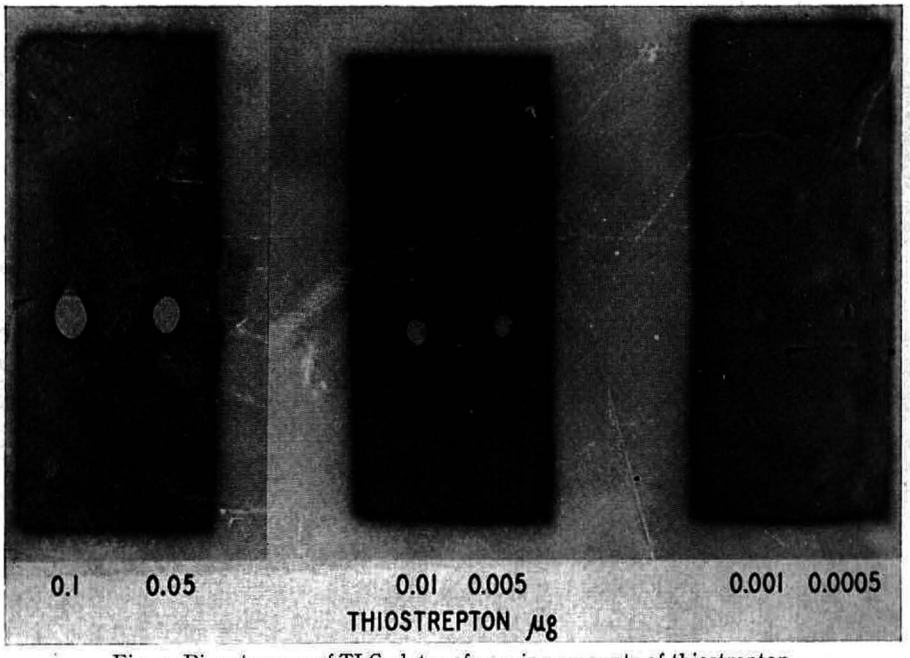


Fig. 1. Bioautogram of TLC plates of varying amounts of thiostrepton.

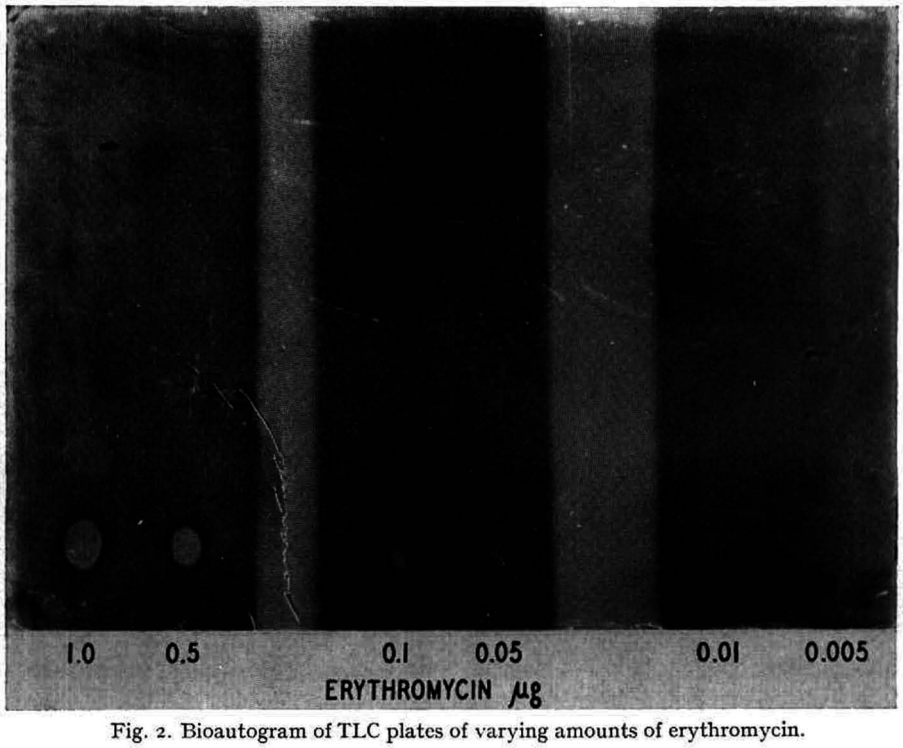


Fig. 2. Bioautogram of TLC plates of varying amounts of erythromycin.

after one hour diffusion time at room temperature, and subsequent incubation at 37° for 16 h. However, this method did not yield consistent results. The procedure as developed with *S. aureus* 209 P was to incubate the chromatographic plate on the seed agar surface for 16 h at 4° to allow diffusion of the antibiotic into the agar. The chromatographic plate and filter paper were removed and the seeded agar examined after an additional overnight incubation at 37°. Sharp, well defined antibiotic zones of inhibition were consistently obtained. Because two overnight incubation periods were inconvenient, a more rapid procedure was sought. *Streptococcus lactis*, facultative in respect to oxygen, was substituted for *Staphylococcus aureus*, and it proved possible to obtain results after overnight incubation at 37° with the chromatographic plate and filter paper lying on the agar surface. Growth of the organism occurred only under the area covered by the glass plate. Sharp, well defined antibiotic zones of inhibition were easily obtained.

The sensitivity of this technique is of the same order of magnitude as that obtained with paper chromatography. Figs. 1 and 2 show the size of the inhibition zones when graded amounts of thiostrepton and erythromycin respectively were chromatographed by TLC and bioautographed against *S. lactis*. As little as 0.001 μg thiostrepton and 0.05 μg erythromycin were detected.

With suitable indicator organisms, antibiotics active against gram negative organisms and antibiotics active against yeast and fungi could be detected. For example, *Escherichia coli* was used to detect polymyxin B sulfate and *Saccharomyces cerevisiae* served as a useful organism for the bioautography of nystatin.

Basic alumina, neutral alumina and silica gel posed no problems in bioautography. None of these adsorbents showed any adverse effect upon growth of the indicator organisms and concomitant reduction of the tetrazolium dye. Acid washed alumina, on the other hand, inhibited the growth of the organisms. With the agar seeded with bacteria, the inhibition was overcome by incorporation of either 0.1 *M* Tris buffer or 0.1 *M* K_2HPO_4 into both Base and Seed Agar, and adjusting the pH to 7.5 prior to sterilization. With agar seeded with the yeast, incorporation of 0.1 *M* KH_2PO_4 into the medium and adjustment of the pH to 6.0 prior to sterilization resulted in good growth under the chromatographic plate.

These bioautographic techniques for spread-layer chromatograms are proving very satisfactory. The convenience of spread-layer chromatography and the specificity and sensitivity of bioautography makes them valuable tools in our studies of antibiotics.

Squibb Institute for Medical Research,
New Brunswick, N.J. (U.S.A.)

EDWARD MEYERS
DOROTHY A. SMITH

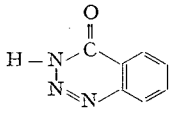
[1] B. J. R. NICOLAUS, C. CORONELLI AND A. BINAGHI, *Farmaco (Pavia), Ed. Prat.*, 16 (1961) 349.
[2] P. A. NUSSBAUMER, *Pharm. Acta Helv.*, 37 (1962) 65.
[3] H. BICKEL, E. GÄUMANN, R. HÜTTER, W. SACKMAN, E. VISCHER, W. VOSER, A. WETTSTEIN AND H. ZÄHNER, *Helv. Chim. Acta*, 45 (1962) 1396.
[4] P. A. NUSSBAUMER, *Pharm. Acta Helv.*, 38 (1963) 245.
[5] T. F. BRODASKY, *Anal. Chem.*, 35 (1963) 343.
[6] T. IKEKAWA, F. IWAMI, E. AKETA AND H. UMEZAWA, *J. Antibiotics (Tokyo)*, Ser. A 16 (1963) 56.
[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] J. DAVÍDEK AND J. BLATTNÁ, *J. Chromatog.*, 7 (1962) 204.

Received August 2nd, 1963

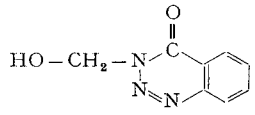
Dünnschichtchromatographischer Nachweis von störenden Verbindungen in der Bestimmung des Dimethyl-dithiophosphorsäureesters des N-Methyl-benzazimids

Die von MEAGHER und Mitarbeitern[1] veröffentliche Methode zur Bestimmung von Rückständen des Dimethyl-dithiophosphorsäureesters des N-Methyl-benzazimids, ein Pflanzenschutzmittel welches unter den Namen Gusathion, Cotnion, Guthion im Handel bekannt ist, wurde mit Erfolg auch von andere Autoren[2,3] angewandt. Die Methode (Zersetzung des Wirkstoffes mit 1 *N* NaOH zum Natrium-anthranilat, Ansauerung mit HCl, Diazotierung, Verkupplung mit N-(1-Naphthyl)-äthylendiamin-dihydrochlorid und kolorimetrische Bestimmung des gebildeten Farbstoffes) ist auch zur Bestimmung des Wirkstoffes in formulierten Produkten mit sehr gut reproduzierbaren Resultaten anwendbar.

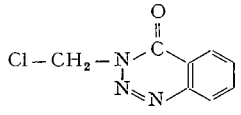
MEAGHER und Mitarbeiter[1] geben an, dass in der Rückstandsanalyse die eventuellen Abbaustoffe wie das Benzazimid (4-Oxo-3-H-1,2,3-benzotriazin) (I), das Hydroxymethyl-benzazimid (4-Oxo-3-hydroxymethyl-1,2,3-benzotriazin) (II), keine interferierende Wirkung in der Bestimmung haben. Bei der Untersuchung des Verhaltens anderer Benzazimid-Derivaten konnten wir feststellen dass im Falle des Chlormethyl-benzazimids (4-Oxo-3-chlormethyl-1,2,3-benzotriazin) (III), welches unter Umständen als Begleitstoff auftreten kann, eine interferierende Wirkung vorliegt: zum Unterschied vom Hydroxymethyl-benzazimid (II) und dem Benzazimid (I),



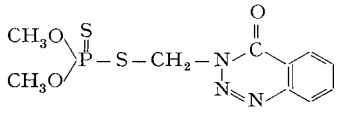
I



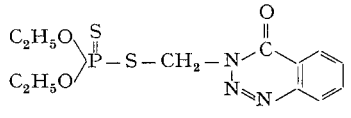
II



III



IV



V

wird das Chlormethyl-Derivat (III) von 1 *N* NaOH in den Bedingungen der MEAGHER'schen Arbeitsvorschrift, zum Anthranilat zersetzt und somit bewirkt seine Gegenwart erhöhte Resultate in der Bestimmung des Wirkstoffes. Den Nachweis dieses eventuellen Begleitstoffes, wie auch anderer Begleitstoffe des Pflanzenschutzmittels konnten wir dünnschichtchromatographisch erbringen. Bei der Verwendung des von BÄUMLER UND RIPPSTEIN[4] für Thiophosphorsäureester vorgeschlagenen Fliessmittels (Hexan–Aceton (4:1)) gelang es uns eine zufriedenstellende Auftrennung eines von uns dargestellten Gemisches des Wirkstoffes und der eventuellen Begleitstoffe zu erzielen. Mit dem gleichen Fliessmittel wird unterschieden, wie auf Grund von der Mitteilungen von BAÜMLER UND RIPPSTEIN[4] auch zu erwarten war, der Dimethyl-dithiophosphorsäureester (IV) vom Diäthyl-dithiophosphorsäureester (V) des N-Methyl-benzazimids, also das Methyl- vom Äthyl-Derivat des Wirkstoffes,

welche ebenfalls als Störstoffe, einer bei der kolorimetrischen Bestimmung des andern[1], auftreten können.

Wir verwendeten mit Kieselgel G beschichtete Platten. Zum Sichtbarmachen der Wirkstoffe und eventuellen Begleitstoffe wurden die Platten nach einer Laufzeit von 45 Min. getrocknet und mit N-Phenyl-2-naphthylamin[5–8], oder, nach entsprechender Vorbehandlung, mit N-(1-Naphthyl)-äthylendiamindihydrochlorid[1,9] besprüht. Diese Reaktion liefert, für sämtliche besprochene Verbindungen, rotviolette bezw. intensiv violette Flecken. Das Benzazimid (I) und das Hydroxymethylbenzazimid (II) welche bei der kolorimetrischen Bestimmung des Wirkstoffes nicht stören, werden auf der Platte, unter den härteren Bedingungen der grossen NaOH-Konzentration und Hitze, unter Aufspaltung des Benzazimid-Ringes, ebenfalls zu Anthranilsäure zersetzt und sind aus diesem Grunde in obiger Weise anfärbbar.

Das Methyl- (IV) und Äthyl-Derivat (V) des Wirkstoffes können, zwecks besserer Differenzierung, ausser ihren R_F-Werten, auch auf anderer Weise von den Benzazimid-Derivaten (I, II und III) unterschieden werden. Zu diesem Zweck werden die zu untersuchenden Stoffe in zwei identischen Gruppen, jede auf einer Hälfte einer 20 × 20 cm Platte aufgetragen. Die Vorbehandlung ist identisch für die ganze Platte zwecks Anfärbung nach Variante II (siehe unten), bis nach dem Besprühen mit 3 *N* HCl. Die Benzazimid-Derivate (I, II und III) liegen dann als Anthranilsäure vor, die Wirkstoffe (IV) und (V) als ein Gemisch von Anthranilsäure und Dimethyl- oder Diäthyl-dithiophosphorsäure und sind somit zu dem von FISCHER UND KLINGELHÖLLER[10,11], zwecks toxikologischem Nachweis, benütztem Spaltstück gebracht worden.

Wenn wir nun auf der einen Hälfte der Platte (während die andere Hälfte mit einer Glasscheibe geschützt ist) die Behandlung, nach Vorschrift, mit $NaNO_2$ und N-Naphthyl-äthylendiamindihydrochlorid fortsetzen und dann die andere Hälfte mit dem von FISCHER UND KLINGELHÖLLER vorgeschlagenem Reagens (3 % Natriumazid in einer *N*/10 Jodlösung) behandeln, erscheinen auf der einen Hälfte die intensiven violetten Flecken sämtlicher Verbindungen auf weissem Grund und auf der zweiten Hälfte nur die weissen Flecken der schwefelhältigen Wirkstoffe auf gelbem Grund.

Die R_F-Werte der verschiedenen Substanzen sind in der Tabelle I angegeben. Die Verbindungen (I) und (II) zeigen identische Werte. Da sie in der kolorimetrischen Bestimmung nicht stören, wurden keine weiteren Fliessmittel zwecks Auf-

TABELLE I

R_F-WERTE VON BENZAZIMID UND DERIVATEN

Substanz	R_F
4-Oxo-3-H-1,2,3-benzotriazin (I)	0.30 ± 0.03
4-Oxo-3-hydroxymethyl-1,2,3-benzotriazin (II)	0.30 ± 0.03
4-Oxo-3-chlormethyl-1,2,3-benzotriazin (III)	0.65 ± 0.04
Dimethyl-dithiophosphorsäureester des N-Methyl-benzazimids (IV)	0.44 ± 0.04
Diäthyl-dithiophosphorsäureester des N-Methyl-benzazimids (V)	0.56 ± 0.04

trennung untersucht. Es empfiehlt sich auf dem Chromatogramm Testsubstanzen parallel laufen zu lassen.

Praktische Durchführung

Es wurden Platten von 100 × 200 mm und 200 × 200 mm verwendet. Die Beschichtung erfolgte mit Kieselgel G nach Stahl (E. Merck, Darmstadt) mit einem Desaga-Streichgerät. Schichtdicke 0.25 mm. Fliessmittel: Hexan–Aceton (4:1).

Variante I. Nach einer Laufzeit von 45–50 Min. wurden die Platten bei 70° getrocknet und mit N-Phenyl-2-naphthylamin besprüht (ca. 1 g N-Phenyl-2-naphthylamin werden in 5 ml Aceton gelöst und dann mit 40 ml Essigsäure 96 % und 15 ml HCl 30 % verdünnt).

Variante II. Nach obiger Laufzeit und Trocknen wurden die Platten mit 1 *N* NaOH besprüht, bei 70° getrocknet, mit 3 *N* HCl besprüht, bei 70° getrocknet, mit 0.2 %-iger Natriumnitritlösung in *N*/10 HCl besprüht, bei 60° getrocknet um schliesslich mit 0.2 % wässriger Lösung von N-Naphthyl-äthylendiamindihydrochlorid besprüht und angefärbt zu werden.

Die untere Nachweisgrenze für beide Anfärbungen ist 1–2 γ.

Sichtbarmachen der Thiophosphorsäureester mit Natriumazid. Nach Besprühen mit 3 *N* HCl (Variante II) und Trocknen bei 70° wird das Chromatogramm mit einer Lösung von 3 % Natriumazid in *N*/10 Jodlösung besprüht.

Die Autoren danken der Direktion der Makhteshim Chemical Works für die Erlaubnis zur Veröffentlichung und Herrn E. GLOTTER für die Einführung in die Dünnschichtchromatographie.

Makhteshim Chemical Works Ltd.,
Beer-Sheva (Israel)

D. KATZ
I. LEMPERT

[1] W. R. MEAGHER, J. M. ADAMS, C. A. ANDERSON UND D. MACDOUGALL, *J. Agr. Food Chem.*, 8 (1960) 282.
[2] S. W. COX, *J. Assoc. Offic. Agr. Chemists*, 44 (1961) 188.
[3] S. W. COX, *J. Assoc. Offic. Agr. Chemists*, 45 (1962) 406.
[4] J. BÄUMLER UND S. RIPPSTEIN, *Helv. Chim. Acta*, 44 (1961) 1162.
[5] O. WOLLENBERG UND G. SCHRADER, *Angew. Chem.*, 68 (1956) 41.
[6] O. WOLLENBERG, *Angew. Chem.*, 68 (1956) 581.
[7] IWAO KAWASHIRO UND TAGEUCHI HIDENAGA, *Bull. Nat. Hyg. Lab.*, No. 76 (1958) 59.
[8] ISAO SATO, *Kumamoto Med. J.*, 14 (1961) 1; *C.A.*, 55 (1961) 24904a.
[9] *Chromatographie*, Firmenschrift E. Merck, S. 161.
[10] R. FISCHER UND W. KLINGELHÖLLER, *Arch. Toxikol.*, 19 (1961) 119.
[11] R. FISCHER UND W. KLINGELHÖLLER, *Pflanzenschutz Ber.*, 11/12 (1961) 165.

Eingegangen den 12. August 1963

Séparation des acides aminés par chromatographie en couche mince de diéthylaminoéthyl-cellulose

Depuis l'introduction par STAHL[1] de la chromatographie en couche mince dans la pratique courante cette méthode s'est rapidement imposée dans divers domaines de la chimie analytique[2,3]. L'avantage de cette technique est son caractère micro-analytique – des quantités de l'ordre de 0.5 μg d'un aminoacide peuvent être révélées – et sa rapidité d'exécution.

Plusieurs supports ont été proposés pour la séparation des acides aminés: alumine[4], silicagel[5], cellulose[6] et dextrannes[7,8].

Dans la présente note, nous apportons quelques résultats obtenus dans la séparation des aminoacides en couche mince de diéthylaminoéthyl (DEAE)-cellulose. Ce support a été déjà utilisé par KNIGHT[9,10] sous forme de papier avec des tampons aqueux comme liquides de développement. La chromatographie sur DEAE-cellulose, appliquée en couche mince sur une plaque de verre et avec des solvants organiques comme moyen de développement, permet la séparation de tous les aminoacides courants avec la même simplicité et rapidité que sur d'autres supports. De plus, sur DEAE-cellulose, la ninhydrine donne avec certains aminoacides des colorations spécifiques permettant de les identifier facilement; d'autre part la sensibilité de la révélation par ce réactif atteint 0.05 μg pour la plupart de ces derniers.

Partie expérimentale

Préparation des plaques. La dimension des plaques de verre est de 20 × 20 cm et accessoirement de 13 × 16 cm. Le nettoyage préalable de ces plaques par un détergent tout d'abord, par la soude caustique ensuite, est indispensable pour obtenir une bonne adhérence du support.

La DEAE-cellulose est une préparation MN 300 DEAE*. Selon les indications du fabricant, on mélange 8 g de poudre MN 300 DEAE avec 2 g de poudre MN 300 (poudre de cellulose) et 90 ml d'eau distillée. La suspension peut être étalée sur la plaque, soit au moyen d'un appareil approprié, soit simplement au moyen d'une spatule. La fluidité de la suspension permet en effet, en inclinant rapidement la plaque tenue sur une main, tantôt d'un côté tantôt de l'autre, d'obtenir un étalement régulier de la couche. Les plaques sont séchées à température ambiante sur une surface parfaitement horizontale.

Les meilleurs résultats sont obtenus lorsqu'on lave la chromatoplaque au préalable en faisant une "chromatographie à blanc" dans le système de solvants utilisé. Quand le solvant atteint le bord supérieur de la chromatoplaque, on la sèche, et après l'application des échantillons à analyser, on l'utilise dans le même sens qu'au cours du lavage. Ce traitement préalable s'avère indispensable pour les systèmes de solvants contenant comme composant un acide organique; dans ce dernier cas, la DEAE-cellulose doit être saturée par l'acide. Le traitement indiqué ci-dessus peut être remplacé par un lavage rapide de la chromatoplaque au moyen d'une solution de l'acide à 20% dans le méthanol.

Les échantillons à analyser sont appliqués de la manière habituelle. Des quantités

* Ets. Macherey, Nagel & Co, Düren, Allemagne.

comprises entre 0.05 et 2 μg de chaque aminoacide peuvent être utilisées. Les points d'application des échantillons doivent être soigneusement séchés.

Le développement se fait toujours dans le sens ascendant. On peut prolonger la chromatographie en adaptant sur le bord supérieur de la chromatoplaque une bande de papier Whatman No. 1 de la même largeur. Cette bande est appliquée contre la couche au moyen d'une plaque de verre de 3 × 20 cm, tenue à ses deux bords par des pinces à dessin. La bande de papier qui prolonge ainsi la couche, passe sous le couvercle et sort de la cuve de chromatographie sur une longueur de quelques centimètres. Ce dispositif permet l'évaporation du solvant au fur et à mesure qu'il arrive dans cette zone.

Il est possible de faire la chromatographie bidimensionnelle sur ce support à condition d'utiliser soit une paire de systèmes ne contenant pas d'acide, soit deux systèmes successifs contenant comme composant le même acide.

Solvants. Parmi la douzaine de systèmes de solvants examinés, 9 ont été retenus:

1. Propanol–ammoniaque 2 *N* (80:20, v/v). Pour éviter que certains acides aminés ne donnent deux taches (leucine, phénylalanine, etc.), avant le développement la chromatoplaque doit être passée au-dessus d'un flacon d'ammoniaque concentrée, ou bien équilibrée pendant 1 ou 2 h dans la cuve avec les vapeurs des solvants utilisés. L'emploi du butanol à la place du propanol donne le même type de séparation entre les acides aminés mais avec des R_F plus petits.

2. Isopropanol–ammoniaque 2 *N* (80:20, v/v). Mêmes précautions que pour système 1.

3. Butanol secondaire–butanol tertiaire–méthyléthylcétone–eau (1:1:1:1, v/v), en présence de 0.5% de diéthylamine. Les mêmes précautions que pour système 1.

4. Pyridine–eau (80:20, v/v). Le lavage préalable de la chromatoplaque par le système de développement, ainsi que nous l'avons indiqué ci-dessus, est recommandé afin d'éviter des traînées.

5. Méthanol–pyridine–eau (80:4:20, v/v).

6. Butanol–acide acétique–eau (4:1:5, v/v), phase supérieure. Le traitement préalable de la chromatoplaque par le système de développement, ou bien par le méthanol contenant 20% d'acide acétique est indispensable (voir ci-dessus).

7. Méthyléthylcétone–pyridine–acide acétique–eau (70:15:2:15, v/v). Voir système 6 pour le traitement préalable de la chromatoplaque.

8. Pyridine–acide acétique–eau (30:10:7, v/v). Voir système 4 pour le traitement préalable de la chromatoplaque.

9. Méthanol–pyridine–acide acétique–eau (80:4:1:20, v/v). Voir système 6 pour le traitement préalable de la chromatoplaque.

Valeurs R_F. Dans le Tableau I nous avons réuni les R_F des aminoacides dans les solvants cités ci-dessus. Pour faciliter la comparaison entre les R_F obtenus dans les divers solvants, nous avons préféré disposer les aminoacides dans l'ordre dans lequel ils apparaissent en général sur une chromatoplaque (l'ordre des R_F décroissants).

Discussion

La chromatographie des aminoacides en couche mince de DEAE-cellulose présente quelques avantages sur celles utilisant d'autres supports: elle apparaît plus sensible et permet l'obtention de colorations spécifiques pour certains aminoacides révélés à la ninhydrine, ce qui facilite leur identification. Il semble que cette coloration spécifique

TABLEAU I

VALEURS R_F ET COLORATIONS À LA NINHYDRINE DES AMINOACIDES SUR COUCHE MINCE DE DEAE-CELLULOSE

(Voir le texte pour la composition des systèmes de solvants. Une flèche indique que la coloration primitive évolue après quelques heures.)

Acide aminé	*R_F × 100 et la coloration à la ninhydrine**								
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9
DL-Leucine	52 (b.)	46 (vi.)	42 (b.)	54 (b.)	75 (b.)	55 (vi.)	36 (vi.)	65 (vi.)	70 (b.)
L-(+)-Isoleucine	48 (b.)	40 (vi.)	39 (b.)	53 (b.)	73 (b.)	51 (vi.)	31 (vi.)	63 (vi.)	70 (b.)
L-(—)-Phénylalanine	44 (b.g.)	35 (vi.g.)	40 (g.f.)	52 (b.g.)	62 (vi.g.)	45 (vi.r.)	42 (vi.r.)	61 (vi.g.)	62 (b.g.)
L-(+)-Valine	40 (b.vi.)	33 (vi.)	28 (b.)	45 (vi.)	69 (b.)	39 (vi.)	21 (vi.)	56 (vi.)	66 (b.)
L-(—)-Méthionine	35 (b.)	29 (vi.)	30 (b.)	48 (b.vi.)	62 (b.)	37 (vi.)	30 (vi.)	52 (vi.b.)	59 (b.)
DL-Proline	30 (j.→v.→g.)	27 (j.→v.→g.)	22 (j.)	33 (j.)	66 (j.)	26 (j.)	14 (j.→v.→g.)	36 (j.)	58 (j.)
L-(—)-Tryptophane	28 (b.g.)	—	35 (b.g.)	46 (b.)	—	36 (vi.g.)	—	—	45 (b.g.)
L-(—)-Tyrosine	22 (b.g.)	—	28 (b.g.)	48 (b.)	61 (b.)	29 (vi.g.)	31 (vi.g.)	57 (vi.)	54 (b.g.)
L-(+)-Alanine	22 (b.vi.)	20 (vi.)	17 (b.)	30 (vi.)	55 (b.)	23 (b.vi.)	10 (vi.)	36 (vi.)	57 (b.)
DL-Thréonine	14 (b.vi.)	17 (vi.)	22 (b.)	33 (vi.)	55 (b.)	19 (b.vi.)	11 (vi.)	38 (vi.)	50 (b.)
DL-Sérine	11 (b.vi.)	8 (vi.)	11 (b.vi.)	28 (vi.)	50 (b.)	14 (b.vi.)	5 (vi.)	26 (b.)	45 (b.)
L-(+)-Glutamine	8 (b.vi.)	20 (vi.)	11 (vi.)	21 (vi.)	47 (vi.)	15 (b.vi.)	6 (vi.)	26 (b.vi.)	43 (b.)
L-(+)-Glutamique (acide)	2 (b.r.)	3 (b.r.)	2 (b.r.)	0 (b.r.)	3 (b.)	14 (b.r.)	0 (b.)	26 (b.)	14 (b.r.)
Glycocolle	12 (g.→g.vi.)	11 (b.g.)	10 (g.f.)	18 (b.g.)	47 (b.g.)	17 (g.b.)	7 (g.)	22 (g.b.)	42 (b.g.)
L-(—)-Histidine·HCl	10 (vi.g.)	11 (vi.g.)	11 (g.)	21 (vi.br.)	41 (vi.g.)	22 (vi.g.)	4 (vi.r.)	22 (vi.g.)	48 (vi.g.)
L-(+)-Arginine·HCl	14 (vi.)	10 (vi.)	11 (vi.)	27 (vi.)	71 (vi.)	24 (vi.)	4 (vi.)	23 (vi.)	62 (vi.)
L-(+)-Lysine·HCl	11 (vi.)	11 (vi.)	6 (vi.)	16 (vi.)	69 (vi.)	20 (vi.)	2 (vi.)	18 (vi.)	60 (vi.)
L-(+)-Asparagine	7 (o.)	7 (o.→br.)	8 (o.)	16 (o.)	36 (o.)	13 (o.)	5 (o.)	18 (o.)	36 (o.)
L-(+)-Aspartique (acide)	2 (b.o.)	3 (b.o.)	4 (b.o.)	0 (b.o.)	2 (v.→b.o.)	9 (v.→b.o.)	0 (v.→b.o.)		7 (v.→b.o.)
L-(—)-Cystine	1 (b.g.)					6 (b.g.)			

* Les symboles suivants ont été adoptés pour désigner les couleurs: b. = bleu; b.g. = bleu gris; b.o. = bleu d'outre-mer; b.r. = bleu roi; b.vi. = bleu violacé; br. = brun; g. = gris; g.f. = gris foncé; g.vi. = gris violacé; j. = jaune; o. = orange; v. = vert; vi. = violet; vi.b. = violet bleu; vi.br. = violet brun; vi.g. = violet gris; vi.r. = violet rose.

soit due à la présence dans la DEAE-cellulose d'un groupe aminé substitué. En effet, des colorations spécifiques sont également observées sur un support formé par l'épichlorhydrine triéthanolamine (ECTEOLA)-cellulose, alors qu'elles ne le sont pas dans le cas des supports cellulosiques ne contenant pas de groupes aminés (CM-cellulose) (résultats non publiés).

Laboratoire de Morphologie Expérimentale et Endocrinologie, Collège de France, Paris (France)

PEDRO DE LA LLOSA
COLETTE TERTRIN
MARIAN JUTISZ

1 E. STAHL, *Chemiker Ztg.*, 82 (1958) 232.
2 E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, Göttingen, Heidelberg, 1962.
3 M. JUTISZ ET P. DE LA LLOSA, *Bull. Soc. Chim. France*, (1963) 2913.
4 M. MOTTIER, *Mitt. Gebiete Lebensm. Hyg.*, 49 (1958) 454.
5 A. F. FAHMY, A. NIEDERWIESER, G. PATAKI ET M. BRENNER, *Helv. Chim. Acta*, 44 (1961) 2022.
6 P. WOLLENWEBER, *J. Chromatog.*, 9 (1962) 369.
7 H. DETERMANN, *Experientia*, 18 (1962) 430.
8 T. WIELAND ET H. DETERMANN, *Experientia*, 18 (1962) 431.
9 C. S. KNIGHT, *Nature*, 194 (1962) 90.
10 C. S. KNIGHT, *J. Chromatog.*, 8 (1962) 205.

Reçu le 22 juillet 1963

The use of Sephadex G-25 in partition column chromatography

Cellulose powder column chromatography has been used for the separation of a variety of materials, *i.e.* sugars and amino acids. However, it has not always been as successful in resolution as might have been predicted from the results obtained with paper sheet chromatography. The main difficulty has been the packing of a uniform column. Modifications have been made in the preparation of the cellulose, as well as the use of other column materials such as starch, but these also suffer from the problem of preparing columns which will give reproducible results.

Sephadex, a cross-linked dextran (obtained from Pharmacia, Inc. as Sephadex G-25), has been used very successfully in the fractionation of various biological materials according to their molecular size[1]. This cross-linked dextran has not been as useful for the fractionation of mixtures of individual monosaccharides or amino acids by what is essentially adsorption chromatography[2].

We have used Sephadex G-25 partition column chromatography to separate mixtures of sugars, amino acids and to some extent a purine and a nucleoside. The method utilizes various solvent mixtures previously used for paper partition chromatography of the materials to be separated.

Experimental

The solvents used were: solvent A, butanol-1–acetic acid–water, 62:15:25 (by volume) and solvent B, ethanol–1 *M* ammonium acetate pH 7.5, 7:3 (by volume). To prepare the columns, 5–10 g of dry Sephadex G-25 powder was stirred for 18–24 h with

100 ml of the solvent used for chromatography. The Sephadex G-25 slurry can be stored in the solvent until used. The Sephadex was then poured as a thick slurry into a glass chromatographic tube with a sintered glass disc bottom, containing about 5 ml of solvent. The slurry is allowed to settle to form the column. Depending on the solvent, 5 g of Sephadex powder will make a column of 5 to 10 ml volume. After the column is attached to an automatic fraction collector, the solvent is allowed to descend to the top of the column bed and the sample dissolved in the same solvent is applied. At this point collection of fractions is started; when the sample has all gone into the column, fresh solvent is added carefully so as not to disturb the column bed. Finally the column is connected to a solvent-containing reservoir.

In an experiment to separate 500 μg each of glucose, rhamnose, N-acetylglucosamine and glucosamine, 1 ml fractions were collected from a column with a bed volume of *ca.* 5 ml. Fractions were tested for the presence of sugar by spotting 25 μl aliquots of each tube on paper, and visualized by use of the alkaline silver nitrate reagent described by TREVELYAN[3]. The tubes containing sugar were then chromatographed in solvent A on Whatman No. 1 paper to identify the sugars present. Table I shows a comparison of the results obtained from a 5-ml column and a second column of greater volume (8–10 ml). The results indicate that the R_{Rh}* values obtained are reproducible.

TABLE I

COMPARISON OF SEPARATION WITH SEPHADEX COLUMNS

Sugar	*5 ml column*		*10 ml column*	
	Elution volume (ml)	R_{Rh}*	*Elution volume (ml)*	R_{Rh}*
Rhamnose	11	1.0	36.4	1.0
N-Acetylglucosamine	13	0.84	42.4	0.87
Glucose	21	0.52	64	0.52
Glucosamine·HCl	31	0.35	91.8	0.39

* $R_{Rh} = \frac{\text{Elution volume rhamnose}}{\text{Elution volume compound}}$

The main disadvantage is that the slower moving components, although well separated, tend to tail, giving more diffuse peaks than do the faster moving components.

The larger column (10 ml) was also used to fractionate a mixture of amino acids and amino sugars using solvent A. A mixture of muramic acid (755 μg), alanine (396 μg), glutamic acid (510 μg), glucosamine·HCl (750 μg), and lysine (505 μg) was applied to the column in a volume of 2.0 ml. Fractions of 3.5 ml were collected, and the tubes containing the materials were found by the use of ninhydrin on spots made on paper. The material in each tube was then identified by paper electrophoresis in 2 *N* acetic acid.

Table II gives the peak volumes in which the compounds were eluted from the column. Alanine and glutamic acid were not completely separated but might well

* $R_{Rh} = \frac{\text{Elution volume rhamnose}}{\text{Elution volume compound}}$

have been if smaller fractions had been taken. The volume required to elute glucosamine·HCl from this run and the volume needed in the run previously described in Table I were similar (87.5 and 91.8 ml respectively) indicating the reproducibility of these columns.

TABLE II

ELUTION VOLUMES OF AMINO COMPOUNDS FROM SEPHADEX COLUMN

Amino compound	*Elution volume (ml)*
Muramic acid	38.5
Alanine	45.5
Glutamic acid	49.0
Glucosamine·HCl	87.5
Lysine	119

The order of elution of the compounds from the columns is identical with that found on paper sheet chromatography in the same solvent. Using a column of 5 ml volume and solvent B, a mixture of adenosine and adenine can readily be separated.

The method described using Sephadex G-25 as a packing for a partition chromatographic column seems to be a useful and reproducible method for separation of sugars and amino acids. The capacity of the columns or the effects of other chromatographic solvents, or of varying the degree of cross linking of the Sephadex on the results and efficiency of the elutions have not been determined.

Research Department, CIBA Pharmaceutical Company, Summit, N.J. (U.S.A.) L. D. ZELEZNICK*

[1] P. FLODIN, *Dextran Gels and their Applications in Gel Filtration*, Pharmacia, Uppsala, Sweden, 1962.
[2] B. GELOTTE, *J. Chromatog.*, 3 (1960) 330.
[3] W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444.

Received July 11th, 1963

* CIBA Fellow in Microbiology.

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

HAIS, I. M. AND MACEK, K. (Editors): *Paper Chromatography. A Comprehensive Treatise*, Academic Press, New York and Publishing House of the Czech. Acad. of Sciences, Prague, 1963, 955 pp.

STOCK, R. AND RICE, C. B. F.: *Chromatographic Methods*, Chapman and Hall, London, 1963, 206 pp.

2. FUNDAMENTALS, THEORY AND GENERAL

SOCZEWIŃSKI, E.: (The extraction and R_M and R_F coefficients in certain multicomponent two-phase systems). *Chem. Anal. (Warsaw)*, 8 (1963) 337–346.

3. TECHNIQUES I

DEYL, Z., ROSMUS, J. AND PAVLÍČEK, M.: (Chromatography in a centrifugal field as a rapid method for routine analyses in food industry). *Prumysl Potravin*, 13 (1962) 601–605; *Anal. Abstr.*, 10 (1963) 3891.

EDWARDS, C. H., RICE, J. O., JONES, J., SEIBLES, L., GADSEN, E. L. AND EDWARDS, G. A.: Chromatography of compounds of biological interest on glass fiber, paraffin-coated, and untreated cellulose paper. *J. Chromatog.*, 11 (1963) 349–354 — extensive R_F tables.

KHIN, L. AND SZÁSZ, G.: A new detection method for aromatic compounds. *J. Chromatog.*, 11 (1963) 416–418 — bromine vapour, then 4-dimethylaminoazobenzene indicator; sensitivity of 23 substances tabulated.

NYBOM, N.: A solvent trough for paper chromatography. *Nature*, 195 (1962) 206.

SMITH, I.: The Unikit. An apparatus and manual for the teaching of chromatography and electrophoresis on paper. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 277–280.

SOCZEWIŃSKI, E.: R_M values of some phenols in the system organic non-polar solvent/dimethyl sulphoxide + glycol. *J. Chromatog.*, 11 (1963) 275–277 — linearity between R_M and solvent composition.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

LARSSON, H.: Paper chromatographic characteristics of substituted diphenylethenes. I. *p,p'*-Substitution in the phenyl rings. *J. Chromatog.*, 11 (1963) 331–338 – R_F values of 29 substances.

WYSZYŃSKA, H. AND ZWOLIŃSKI, J.: (Application of chromatography and spectrometry for quantitative determination of tar substances in air pollutants. I. Determination of benzo(*a*)-pyrene). *Roczniki Panstwowego Zakladu Hig.*, 13 (1962) 503–510; *Anal. Abstr.*, 10 (1963) 3931.

6. ALCOHOLS

HROMATKA, O. AND STAINER, J.: (Paper chromatographic determination of glycerol and dihydroxyacetone in vinegar malts and vinegar). *Branntweinwirtschaft*, 102 (1962) 703–704; *C.A.*, 58 (1963) 8387b.

WOLLRAB, V. AND STREIBL, M.: Über die Zusammensetzung der Braunkohle. V. Beitrag zur Papierchromatographie höherer aliphatischer Carbonsäuren und Alkohole. *Collection Czech. Chem. Commun.*, 28 (1963) 1895–1902 — C_{14}–C_{34} aliphatic acids and alcohols.

7. PHENOLS

BLASS, J. (with the assistance of VICAIGNE, M. B.): L'emploi du réactif de Folin en chromatographie et électrophorèse sur papier. *J. Chromatog.*, 11 (1963) 278.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

BHANU, K. U., RAJADURAI, S., SASTRY, K. N. S. AND NAYUDAMMA, Y.: Tannins present in babul leaves. *Bull. Central Leather Res. Inst. Madras (India)*, 9 (1962) 100–104; *C.A.*, 58 (1963) 7053h.

BOITSCHINOFF, A. AND ACHATARDJEFF, C.: Inhaltsstoffe phenolischer Natur in den Blättern von *Rhododendron ponticum* L. *Arch. Pharm.*, 296 (1963) 692–699.

KRISHNAMOORTHY, V. AND SESHADRI, T. R.: Survey of anthocyanins from Indian sources. III. *J. Sci. Ind. Res. (India)*, 21B (1962) 591–593; *C.A.*, 58 (1963) 8235e.

9. OXO COMPOUNDS

CAVILL, G. W. K. AND HINTERBERGER, H.: (Dolichodial and related compounds). *Verhandl. 11. Intern. Kongr. Entomol. Wien, 1960*, pp. 284–289; *C.A.*, 58 (1963) 8258c.

10. CARBOHYDRATES

BEER, J. Z.: Quantitative paper radiochromatography using Tollens reagent. II. Ammoniacal silver nitrate reagents containing less than equimolar proportions of ammonia for the oxidation of saccharides on paper chromatograms. *J. Chromatog.*, 11 (1963) 247–252.

BHOWN, A. S.: Separation and identification of carbohydrates from coconut by circular paper chromatography *Indian J. Appl. Chem.*, 25, No. 2/3 (1962) 97–98; *C.A.*, 58 (1963) 7134c.

BONATTI, A. A., ABUIN, J. C. AND DE GENER, A. C.: (Differential detection of hexoses and pentoses in paper chromatography by means of sulphanilic acid). *Rev. Asoc. Bioquim. Arg.*, 27 (1962) 86–93; *Anal. Abstr.*, 10 (1963) 3721.

BOURNE, E. J., LEES, E. M. AND WEIGEL, H.: Paper chromatography of carbohydrates and related compounds in the presence of benzeneboronic acid. *J. Chromatog.*, 11 (1963) 253–257 — R_F values of 62 sugars in 2 systems.

COOK, G. M. W.: Linkage of sialic acid in the human erythrocyte ultrastructure. *Nature*, 195 (1962) 159–161.

COURTOIS, J. E. AND PERCHERON, F.: Récentes acquisitions sur l'identification chromatographique des oses et oligosaccharides. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 23–40 — chromatography on paper, column and thin layers.

DI FERRANTE, N.: Acid mucopolysaccharides of normal human urine. *J. Lab. Clin. Med.*, 61 (1963) 633–641.

DORCHEUS, S. H. AND WILLIAMS, D. G.: Study of the reactions in the zinc chloride–benzaldehyde–glucose system. *J. Org. Chem.*, 28 (1963) 775–777.

DUTTON, G. G. S. AND TANAKA, Y.: Methylation of sugar dithioacetals. IV. D-Xylose diethyl dithioacetal. *Can. J. Chem.*, 40 (1962) 1899–1902; *C.A.*, 58 (1963) 2494a.

HORST, P. AND MCGLUMPHY, J. H.: (Papierchromatographische Methoden zum Nachweis von Verfälschungen in Vanille-Extrakten). *Ann. Fals. Expert. Chim.*, 55 (1962) 264–273; from *Z. Anal. Chem.*, 197 (1963) 391.

MONTREUIL, J., SPIK, G., CHOSSON, A., SEGARD, E. AND SCHEPPLER, N.: Les procédés d'étude de la structure de glycoprotéides. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 47–64.

MOTQUIN, A.: (Isolierung und Nachweis von Lactose in Nahrungsmitteln). *Ann. Fals. Expert. Chim.*, 54 (1961) 281–283; from *Z. Anal. Chem.*, 197 (1963) 389.

OHKUMA, S.: Chromatographs of some N-acetylhexosamines on paper impregnated with sodium tetraborate. *Proc. Japan. Acad.*, 38 (1962) 562–567; *C.A.*, 58 (1963) 8222e.

ORLOVA, O. K. AND MARYASINA, A. D.: (Separation, identification and quantitative assay of methylated mannose derivatives by paper electrophoresis). *Biokhimiya*, 28 (1963) 577–582 — combination with paper chromatography.

RICHTER, M.: (Paper chromatographic fractionation of starch carbohydrates. I. The value of paper-chromatographic capillary analysis in the fractionation of starch carbohydrates). *Stärke*, 14 (1962) 337–342; *Anal. Abstr.*, 10 (1963) 2895.

SOWDEN, F. J. AND LVARSON, K. C.: Analysis of carbohydrate material in soil. II. Use of cellulose columns and paper chromatography for determining constituent sugars. *Soil Sci.*, 94 (1962) 340–343; *Anal. Abstr.*, 10 (1963) 2945.

STROH, H. H., DOMANN, E. AND HASCHKE, E.: (Separation and identification of substituted sugar hydrazones by paper chromatography). *Z. Chem.*, 2 (1962) 338–339; *Anal. Abstr.*, 10 (1963) 3727.

TIKHOMOLOVA, M. P. AND AIZENSHTAT, E. L.: (Quantitative determination of glucose and maltose by means of ascending paper chromatography). *Vopr. Sovrem. Fiz. i Mat., Akad. Nauk Uz. SSR*, (1962) 120–123; *C.A.*, 58 (1963) 7123f — densitometry after detection with 3,5-dinitrosalicylic acid.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BOLE, G. G.: The incorporation of ^{32}P-labelled orthophosphate and glycerol-1,3-^{14}C into the lipids of the polyvinyl sponge granuloma. *J. Clin. Invest.*, 42 (1963) 787–799.

DAVÍDEK, J.: Rapid detection of gallic acid esters by centrifugal chromatography. *J. Chromatog.*, 11 (1963) 419–420.

FREUDENBERG, K. AND LEHMANN, B.: Radioaktive Isotope und Lignin. X. Untersuchung eines mit ^{14}C markierten Ligninpräparates. *Chem. Ber.*, 96 (1963) 1850–1854 — R_F values of 15 aromatic acids.

HAMDY, M. M. AND GOULD, W. A.: Varietal differences in tomatoes: a study of α-keto acids, α-amino compounds and citric acid in eight tomato varieties before and after processing. *J. Agr. Food Chem.*, 10 (1962) 499–503; *Anal. Abstr.*, 10 (1963) 3906.

KATES, M., CHAN, T. M. AND STANACEV, N. Z.: Aliphatic diether analogs of glyceride-derived lipids. I. Synthesis of D-α,β-dialkyl glyceryl ethers. *Biochemistry*, 2 (1963) 394–397.

KATSCH, G. AND CRAWACK, G.: Phenolcarbonsäuren in menschlichen Nahrungsprodukten. *Med. Ernähr.*, 4 (1963) 1–10 — separation of 34 spots (14 identified).

KWAPNIEWSKI, Z. AND SLIWIOK, J.: The application of fuchsine dyes as developers in paper chromatography of the higher fatty acids. *Chem. Anal. (Warsaw)*, 8 (1963) 433–435.

SCHUMACKER, R.: (Paper chromatography of the lichen acids of *Everniopsis pseudoreticulata*). *Bull. Soc. Roy. Sci. Liege*, 30 (1961) 452–457; *C.A.*, 58 (1963) 8232g.

13. STEROIDS

BIGLIERI, E. G., SATHOSI, HANE, SLATON, P. E. AND FORSHAM, P. H.: *In vivo* and *in vitro* studies of adrenal secretions in Cushing's syndrome and primary aldosteronism. *J. Clin. Invest.*, 42 (1963) 516–524.

CORREAL, L. AND BALESTRERI, R.: (Metabolism of C-16 oxygenated estrogens. Conversion *in vitro* of estriol to 16-keto-17β-estradiol by the liver of the rat). *Arch. "E. Maragliano" Pathol. Clin.*, 18 (1962) 653–658; *C.A.*, 58 (1963) 7100a.

GABBAY, K. H. AND WATERHOUSE, C.: Paper chromatography of cholesterol esters. *J. Chromatog.*, 11 (1963) 241–246.

OAKEY, R. E., STITCH, S. R. AND ECCLES, S. S.: The application of partition chromatography to the separation and characterisation of oestrogens by isotope dilution. *J. Chromatog.*, 11 (1963) 355–363.

PASQUALINI, J. R.: (Identification and separation of 6β,17α,21α-trihydroxypregn-4-ene-3,11,20-trione (6β-hydroxycortisone) in human urine). *Compt. Rend.*, 255 (1962) 3258–3260; *Anal. Abstr.*, 10 (1963) 3405.

PAYNE, A. H. AND MASON, M.: The enzymic synthesis of the sulfate esters of estradiol-17β and diethylstilbestrol. *Biochim. Biophys. Acta*, 71 (1963) 719–724.

THEWALT, K. AND ZIMMERMANN, W.: (Simplified procedure for the routine determination of estrogens in urine by the separation of estriol, estradiol and estrone by paper chromatography). *Acta Endocrinol.*, 38 (1961) 121–127; *C.A.*, 58 (1963) 8197e.

14. STEROID GLYCOSIDES

BRUTON, J., HERMAN, R. H. AND CLAYTON, L. E.: Effect of phosphoric acid on digitalis compounds. *Nature*, 195 (1962) 904.

KUBELKA, W. AND WICHTL, M.: Neue Glykoside aus *Convallaria majalis*. *Naturwissenschaften*, 50 (1963) 498.

SANDOZ, LTD.: (Sterol derivatives). *Belg. Pat.* 612,024, June 27, 1962; Swiss Appl., Dec. 29, 1960, 7 pp.; *C.A.*, 58 (1963) 3495h.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BANNARD, R. A. B. AND CASSELMAN, A. A.: Paper chromatography of ethylenediamine dihydrochloride and diacetate in acidic solvent systems. *Can. J. Chem.*, 41 (1963) 92–107; *C.A.*, 58 (1963) 3297g.

CAMARERO, S. F. J.: (Über die zweidimensionale papierchromatographische Trennung von Metaboliten des Serotonins in Harn). *Laboratorio (Granada)*, 33 (1962) 407–415; from *Z. Anal. Chem.*, 198 (1963) 232.

FRIEDHOFF, A. J. AND VAN WINKLE, E.: A method for the detection of β-phenylethylamines and phenylethylamino acids. *J. Chromatog.*, 11 (1963) 272–274 — 40 substances tried; Ehrlich after ninhydrin.

ICHIBAGASE, H. AND KOJIMA, S.: Synthetic sweetening agents. I. Paper chromatography of cyclamate sodium, dulcin and saccharin sodium. *Yakugaku Zasshi*, 82 (1962) 1616–1620; *C.A.*, 58 (1963) 8357g.

SMITH, P.: Chromatography of some phenolic amines. *Nature*, 195 (1962) 174.

SZÁSZ, G. AND SZÁSZ, M.: (Paper chromatographic detection of ethanolamine and diethanolamine in triethanolamine). *Acta Pharm. Hung.*, 32 (1962) 260–262; *Anal. Abstr.*, 10 (1963) 3738.

WEISS, B. AND ROSSI, G. V.: Separation of catecholamines by paper chromatography. *Nature*, 195 (1962) 178.

18. AMINO ACIDS

GAUTIER, J. A. AND BLANC-GUÉNÉE, J.: Recherches chromatographiques sur les acides aminés libres des vins naturels. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 225–236.

KRITZMAN, M. G., SUKHAREVA, B. S. AND KONIKOVA, A. S.: Sites of incorporation of phenylalanine labelled with carbon-14 into insulin and its chain B *in vitro*. *Nature*, 195 (1962) 600–602.

LAMPERTICO, M. AND PERSANI, G.: (Plasma iodine compounds by paper chromatography in stages of increased thyroid function). *Atti Accad. Med. Lombarda*, 16 (1961) 283–286; *C.A.*, 58 (1963) 8309h.

PEETERS, H., VAN DAMME, D. AND VAN DOREN, J.: Chromatography and electrochromatography of gastric and duodenal extracts. *Chromatographie Symposium II. Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 97–111.

POSTMES, T.: Sensitive, simple quantitative reaction for iodinated amino-acids of human serum on paper chromatograms. *Acta Endocrinol.*, 42 (1963) 153–162; *Anal. Abstr.*, 10 (1963) 2840.

SMITH, J. N.: Detoxications of aromatic acids with glutamic acid and arginine in spiders. *Nature*, 195 (1962) 399–400 — R_F values of 11 amino acid derivatives.

SULLIVAN, W. D. AND LAZARZ, J. S.: Chromatographic studies of amino acids as found in defined medium before and after growth of *Tetrahymena pyriformis*. *Broteria*, 31, No. 2 (1962) 67–79; *C.A.*, 58 (1963) 7161f.

SZANTAI, I.: Behavior of amino acids identified with ninhydrin in a hydrogen chloride atmosphere. II. *Rev. Chim. (Bucharest)*, 10 (1959) 334–337; *C.A.*, 58 (1963) 7124a.

WELLBY, M. L.: Methods for the separation and identification of the iodine-containing amino-acids in human plasma. *Australian J. Exptl. Biol. Med. Sci.*, 40 (1962) 405–413; *Anal. Abstr.*, 10 (1963) 3393.

ZILKHA, A. AND RAPPOPORT, S.: Syntheses of hydroxyalkyl and mercaptoalkyl derivatives of sulfur-containing amino acids. *J. Org. Chem.*, 28 (1963) 1105–1107.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

GRÖSCHEL-STEWART, U. AND TURBA, F.: ^{14}C-Markierung und Peptidkarten der SH-Regionen von Actomyosin, Myosin, Actin und H-Meromyosin. *Biochem. Z.*, 337 (1963) 104–108.

GRÖSCHEL-STEWART, U. AND TURBA, F.: Zuordnung der Adenosintriphosphatase und des Aktinbindungsvermögen des H-Meromycins zu bestimmten SH-Bezirken. *Biochem. Z.*, 337 (1963) 109–114.

ISKIERKO, J.: (Protein fractions of purified and concentrated diphtheria toxoid). *Med. Doswiadczalna Mikrobiol.*, 14 (1962) 323–330; *C.A.*, 58 (1963) 8166b.

KOLOGLU, Y., WIESEL, L. L., POSITANO, V. AND ANDERSON, G. E.: An immune chromatographic method for assay in serum antibodies to glucagon and insulin. *Proc. Soc. Exptl. Biol. Med.*, 112 (1963) 518–523 — ion-exchange resin-impregnated paper.

MAZUR, R. H. AND SCHLATTER, J. M.: A novel peptide cleavage reaction. *J. Org. Chem.*, 28 (1963) 1025–1029.

TONGE, B. L.: Reaction of fluoro-2,4-dinitrobenzene with amino-acids and peptides. *Nature*, 195 (1962) 491.

WIESEL, L. L., POSITANO, V., KOLOGLU, Y. AND ANDERSON, G. E.: Chromatographic separation of glucagon and insulin from serum by resin-exchange paper. *Proc. Soc. Exptl. Biol. Med.*, 112 (1963) 515–518.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

CHATAMRA, B. AND JONES, A. S.: The permanganate oxidation of uracil and cytosine and their 1-substituted derivatives. *J. Chem. Soc.*, (1963) 811–815.

DAVILA, C. AND LEDOUX, L.: Chromatographie des acides désoxyribonucléiques sur papier de diéthylaminoéthyl-cellulose. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 87–96.

FISCHL, J. AND SEGAL, S.: (Papierchromatographische Identifizierung von Barbitursäurederivaten in toxikologischen Fällen). *Clin. Chem.*, 7 (1961) 252–256; from *Z. Anal. Chem.*, 198 (1963) 393.

HORWITZ, J.-P., CHUA, J., URBANSKI, J. A. AND NOEL, M.: Nucleosides. III. 1-(2-Deoxy-3′-5′-epoxy-β-D-*threo*-pentofuranosyl)-thymine. *J. Org. Chem.*, 28 (1963) 942–944.

JACOBSON, K. B. AND NISHIMURA, S.: Paper chromatography of transfer ribonucleic acid on diethylaminoethyl-cellulose. *Biochim. Biophys. Acta*, 68 (1963) 490–493.

MOSS, G. P., REESE, C. B., SCHOFIELD, K., SHAPIRO, R. AND LORD TODD: Nucleotides. XLVII. The catalytic oxidation of nucleosides and nucleotides: a projected stepwise degradation of polynucleotides. *J. Chem. Soc.*, (1963) 1149–1154.

22. ALKALOIDS

BHATTACHARJI, S., ROY, A. C. AND DHAR, M. L.: (Chemical examination of the roots of *Cissampelos pareira*. II. Isolation and structure of hayatinine). *J. Sci. Ind. Res. (India)*, 21B (1962) 428–433; *C.A.*, 58 (1963) 3465e.

COUSSIO, D., MARINI-BETTÒLO, G. B. AND MOSCATELLI, V.: The separation of alkaloids on paper impregnated with zirconium phosphate. *J. Chromatog.*, 11 (1963) 238–240 — 35 alkaloids in 11 solvents.

CULVENOR, C. C. J. AND SMITH, L. W.: Alkaloids of *Crotalaria trifoliastrum* and *C. aridicola*. I. Methyl ethers of supinidine and retronecine. *Australian J. Chem.*, 15 (1962) 121–129; *C.A.*, 58 (1963) 3466d.

NEU, R.: Ein neuer aber farbiger Nachweis von Alkaloiden und organischen Basen mit Tetraphenylbornatrium. *J. Chromatog.*, 11 (1963) 364–368 — visualization by subsequent treatment with fisetin or related flavonols; R_F values of 21 substances.

PARIS, R.: Chromatographie sur papier et en couche mince des alkaloïdes et des flavonoïdes. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 11–22 — a review.

ROUFFIAC, R., LATTES, A. AND VALDIGUIÉ, P.: Détermination des constantes d'ionisation par chromatographie sur papier. *Ann. Pharm. Franc.*, 21 (1963) 413–422.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

ACHESON, R. M. AND KING, L. J.: The metabolism of indole-3-carboxylic acid by the rat. *Biochim. Biophys. Acta*, 71 (1963) 643–649 — R_F values of 30 Ehrlich-positive constituents of rats urine.

BARRETO, R. C. R. AND SABINO, S. A.: Chromatographic studies on isonicotinic acid hydrazide and its metabolic derivatives. III. New solvents and new techniques. *J. Chromatog.*, 11 (1963) 344–348 — elution-concentration method for serum samples.

BERNATH, G. AND KOCZKA, K.: (Correlation between structure and R_F-values of organic substances. I. Paper chromatographic investigation of homologs of N-alkyl-1-methyl-6,7-dimethoxy-3,4-dihydroisoquinolinium iodide and of N-alkyl-1-methyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolinium iodide in the systems C_4–C_8 *n*-alcohols–water). *Acta Chim. Acad. Sci. Hung.*, 31 (1962) 443–448; *C.A.*, 58 (1963) 3392a.

BRÄUNIGER, H. AND KOINE, A.: Eine weitere Darstellungsmethode von N-glykosiden des Benzimidazols. *Arch. Pharm.*, 296 (1963) 668–680.

SHVAYTSMAN, Z. D.: (Paper chromatographic bilirubin determination in acute and chronic damage). *Tr. Leningr. Sanit.-Gigien. Med. Inst.*, 69 (1961) 31–44; *C.A.*, 58 (1963) 7223f.

WAGNER, G. AND PISCHEL, H.: Zur O→N-Umglykosidierung und Anomerisierung von 2-Glykosyloxy-pyridinen und -picolinen. *Arch. Pharm.*, 296 (1963) 699–713 — R_F values of 18 pyridine glycosides.

24. ORGANIC SULPHUR COMPOUNDS

ARAGONÉS-APODACA, R.: (Study of the course of industrial reactions by paper chromatography. Production of gamma acid). *Inform. Quim. Anal. (Madrid)*, 16 (1962) 168–173, 176; *Anal. Abstr.*, 10 (1963) 3749.

DENTI, E., LUBOZ, M. P. AND MASSAGLIA, A.: Paper chromatography of polyphenyls. Qualitative separation of sulphonic acid derivatives of diphenyl and *o*-, *m*- and *p*-terphenyls. *J. Chromatog.*, 11 (1963) 339–343.

FISHMAN, V. AND GOLDENBERG, H.: Metabolism of chlorpromazine. IV. Identification of 7-hydroxychlorpromazine and its sulfoxide and desmethyl derivatives. *Proc. Soc. Exptl. Biol. Med.*, 112 (1963) 501–506.

FOLKARD, A. R. AND JOYCE, A. E.: Separation of sulphidimines of sulphides on paraffin-treated paper. *J. Chromatog.*, 11 (1963) 415–416 — 6 sulphidimines.

MÜTING, D.: Detection and metabolism of hydroxychloroquinsulphate and hydroxychloroquindiphosphate in human beings. *Nature*, 195 (1962) 1003–1004.

NICKERSON, W. J., FALCONE, G. AND STRAUSS, G.: Studies on quinone-thioethers. I. Mechanism of formation and properties of thiodione. *Biochemistry*, 2 (1963) 537–543.

VAN DER VENNE, M. TH. AND T'SIOBBEL, J. B.: Considérations sur l'analyse chromatographique des sulfamides. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 191–202 — chromatography of 38 sulphonamides in 12 solvent systems on paper and in 5 systems in thin-layers of silica gel; quantitative analysis after elution of dyes.

25. ORGANIC PHOSPHORUS COMPOUNDS

FRIEDRICH, W.: Zur Kenntnis der Phosphorsäureester der Benzimidazolriboside. *Z. Naturforsch.*, 18b (1963) 455–462 — R_F values of 11 phosphates.

ROBINSON, J. R.: Technique for neutron activation of phosphorus compounds on paper chromatograms. *Can. J. Biochem. Physiol.*, 40 (1962) 1460–1463; *Anal. Abstr.*, 10 (1963) 2971.

27. VITAMINS

EBERHART, B. M. AND TATUM, E. L.: Thiamine metabolism in wild-type and mutant strains of *Neurospora crassa*. *Arch. Biochem. Biophys.*, 101 (1963) 378–387 — bioautography using macroconidia of thiamine-less *Neurospora* strains.

MÜLLER, O. AND MÜLLER, G.: Zur Chemie und Biochemie der "Cobalamine". XXIV. Weitere Synthesen von Corrinod-Coenzym-Analogen und Umwandlung einiger dieser Derivate durch *Propionibacterium shermanii*. *Biochem. Z.*, 337 (1963) 179–194 — R_F values of 12 cobalamines.

ZIEGLER, I.: Di- und Tetrahydropterin bei der Blaualge *Anacystis nidulans*. *Biochem., Z.*, 337 (1963) 62–68.

28. ANTIBIOTICS

BATCHELOR, F. R., COLE, M., GAZZARD, D. AND ROLLINSON, G. N.: Penicillin-like substances in preparations of 6-aminopenicillanic acid. *Nature*, 195 (1962) 954–955.

MILLER, R. P.: A paper chromatographic assay for cephalosporins. *Antibiot. Chemotherapy*, 12 (1962) 689–693; *C.A.*, 58 (1963) 7122h.

30. SYNTHETIC AND NATURAL DYES

ARIENT, J. AND HAVLÍČKOVÁ, L.: Imidazol-Farbstoffe. VIII. Reaktion von substituierten Phtalsäureanhydrid mit 1,2-Diaminobenzol. *Collection Czech. Chem. Commun.*, 28 (1963) 1885–1893 — R_F values of 20 compounds.

FUJITA, M., FURUYA, T. AND MATSUO, M.: Metabolism of naturally occurring anthraquinones. III. Metabolism of alizarin dimethyl ether. *Chem. Pharm. Bull. (Tokyo)*, 10 (1962) 909–911; *C.A.*, 58 (1963) 8298e.

ROMANOWSKI, R. D., KUC, J. AND QUACKENBUSH, F. W.: Biochemical changes in seedlings of bean infected with *Collectotrichum lindemuthianum*. *Phytopathology*, 52 (1962) 1259–1264; *C.A.*, 58 (1963) 7142e.

TYIHAK, E.: Active ingredients of red beets. Preliminary reports. *Sci. Pharm.*, 30 (1962) 185–187; *C.A.*, 58 (1963) 7135g.

WAGNER, H., HÖRHAMMER, L., LEUE, H. J. AND KÖNIG, H. W.: (New methods in pharmacognostical instruction. III. Chromatography of the constituents of rhubarb rhizome and its pharmaceutical preparations). *Deut. Apotheker-Ztg.*, 102 (1962) 1278–1280; *Anal. Abstr.*, 10 (1963) 3416.

WEISS, J. B. AND SMITH, I.: Spectrophotometric identification of chromatographically separated plastid pigments. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 293–297.

33. INORGANIC SUBSTANCES

ALBERTI, G. AND MASSUCCI, M. A.: Chromatographic separation and identification of some individual rare earths on paper by means of fluorescence. *J. Chromatog.*, 11 (1963) 394–400 — on Amberlite SA-2 ion-exchange paper.

BAYKUT, F. AND KÜCÜKUYSAL, A.: (New paper-chromatographic determination of cupric ions). *Istanbul Univ. Tip Fak. Mecmuasi*, 27 (1962) 26–31; *Anal. Abstr.*, 10 (1963) 2599.

COHEN-NORMANN, M. E.: Dispositif simple de chromatographie de partage ascendante sur papier. Application á la détection du cadmium. *Bull. Soc. Chim. France*, (1963) 320.

FISEL, S. AND GIURGIU, D.: (Papierchromatographisches Verhalten von Phenoxyacetaten von Mangan, Kobalt, Nickel, Kupfer, Zink und Cadmium). *An. Sti. Univ., Iasi, N.S., Sect. 1*, (1960) 975–976; from *Z. Anal. Chem.*, 198 (1963) 372.

JANJIĆ, T. J., ĆELAP, M. B. AND ŠPANOVIČ, Ž. F.: (Determination of small amounts of mercury, silver, platinum, palladium and gold on paper impregnated with cadmium sulphide). *Glasnik Hem. Drustva, Beograd*, 25–26 (1960–1961) 531–534; *Anal. Abstr.*, 10 (1963) 3068.

MOGHISSI, A. AND EDGÜER, E.: Papierchromatographische Trennung von Zirkon und Niob. *J. Chromatog.*, 11 (1963) 389–393.

MORETTE, A. AND DJAVAD-NIA, D.: Sur la séparation chromatographique sur papier des ions: magnésium, calcium, sodium et potassium. Application aux eaux minérales. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 65–74.

PANG, SHU-WEI, LIANG, SHU-CHUAN: Separation of lanthanides, scandium and thorium by means of paper chromatography. *Acta Chim. Sinica*, 28 (1962) 148–154; *Anal. Abstr.*, 10 (1963) 3146.

PFEIL, E.: Papierchromatographische Trennung der gebräuchlichen Kationen. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 203–206 — separation of 34 cations into groups using the classical scheme and then paper chromatography.

POLLARD, F. H., NICKLESS, G. AND ROTHWELL, M. T.: Chromatographic studies on the hydrolysis of phosphorus compounds. VII. The hydrolysis of phosphorus trihalides and pseudohalides. *J. Chromatog.*, 11 (1963) 383–388.

RUDOLFF, H.: (Paper chromatographic detection of alkali metals). *Deut. Apotheker-Ztg.*, 102 (1962) 1571–1572; *Anal. Abstr.*, 10 (1962) 3596.

34. RADIOACTIVE COMPOUNDS

MAGNUSSON, G.: Purity and stability of commercial sodium iodohippurate labelled with iodine-131. *Nature*, 195 (1962) 591–592.

Thin-layer Chromatography

3. TECHNIQUES I

AMENTA, J. S.: Use of sequentially applied location reagents and multiple transparent overlays in thin-layer chromatography. *J. Chromatog.*, 11 (1963) 263–264.

DAUVILLIER, P.: Chromatographie préparative sur plaques. Confection d'un mélange (gel de silice–plâtre) permettant un étalement homogène de couches épaisses et leur séchage sans craquelures. Application au moyen d'un nouvel appareil. *J. Chromatog.*, 11 (1963) 405–407 — retardation of solidification by preheating to 500° for 4 h, decrease of $CaSO_4$ content to 5% and slow drying.

MARCUCCI, F. AND MUSSINI, E.: A new apparatus for thin-layer chromatography. *J. Chromatog.*, 11 (1963) 270–271 — the plate progresses at a constant speed during spreading.

RAHANDRAHA, T.: Utilisation de la poudre de verre en chromatographie sur couche mince. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 261–276.

WOHLLEBEN, G.: The adsorption system in column and thin-layer chromatography. Recent results. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques. Bruxelles, 1962*, publ. 1963, pp. 281–283 — discussion of the standardization.

WOHNLICH, J. J.: Chromatographie sur verre sablé et muni de couches minces. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 255–260 — chromatography of amino acids, sugars and lipids on glass layers.

7. PHENOLS

AURENGE, J., DEGEORGES, M. AND NORMAND, J.: Séparation et dosage par chromatographie sur couche mince de différents constituants issus de la synthèse du diphénylolpropane. *Bull. Soc. Chim. France*, (1963) 1732–1739.

SCHORN, P. J.: Dünnschichtchromatographie von Phloroglucinbutanonen. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, Summary, p. 307.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

HÄNSEL, R., LANGHAMMER, L., FRENZEL, J. AND RANFT, G.: Dünnschichtchromatographische Untersuchungen an Anthochlor-Pigmenten. Vergleich des natürlichen Bractein-Aglykons mit synthetischem 4,6,3′,4′,5′-Pentaoxy-auron. *J. Chromatog.*, 11 (1963) 369–375 — R_F values of 11 aurones and 10 chalcones.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

GOEBELL, H. AND KLINGENBERG, M.: Dünnschichtchromatographie von Tricarbonsäurezyklus-Substraten mit Autoradiographie. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 153–165 — two-dimensional chromatography on cellulose layers; quantitative analysis after elution.

RONKAINEN, P.: Die Dünnschichtchromatographie der Ketosäuren. *J. Chromatog.*, 11 (1963) 228–237 — R_F values of 8 keto acids.

13. STEROIDS

CHAMBERLAIN, J. AND THOMAS, G. H.: R_M values in adsorption chromatography. *J. Chromatog.*, 11 (1963) 408–409 — R_F values of 19 steroids.

15. TERPENE DERIVATIVES

JORK, H.: Die Dünnschicht-Chromatographie als Analysenmethode zur Untersuchung lipophiler sekundärer Pflanzeninhaltsstoffe unter besonderer Berücksichtigung der Harze und Balsame. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 213–223 — Silica gel G impregnated with paraffin oil or silicone oil; 19 various resins.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

COFFEY, R. G. AND NEWBURGH, R. W.: The effect of calcium sulfate as the binder in DEAE-cellulose thin-layer chromatography for separating nucleic acid degradation products. *J. Chromatog.*, 11 (1963) 376–382 — improvement with suitable amounts of $CaSO_4$.

DYER, T. A.: The separation of the nucleotides of an alkaline hydrolysate of ribonucleic acid by thin-layer chromatography. *J. Chromatog.*, 11 (1963) 414–415 — on DEAE cellulose.

MEHAPATRA, G. N. AND FRIEDMAN, O. M.: Separation of isomeric methylated deoxyguanosines on thin cellulose layers prepared with a glass rod applicator. *J. Chromatog.*, 11 (1963) 265–266.

22. ALKALOIDS

IKRAM, M., MIANA, G. A. AND ISLAM, M.: Chromatographic separation of *Rauwolfia serpentina* and opium alkaloids on thin layers of alumina. *J. Chromatog.*, 11 (1963) 260–262 — R_F values of 9 alkaloids.

PARIS, R. R. AND PARIS, M.: Chromatographie en couche mince des alcaloïdes et des pigments anthocyaniques. *Bull. Soc. Chim. France*, (1963) 1597–1599.

25. ORGANIC PHOSPHORUS COMPOUNDS

WAGNER, H.: Dünnschichtchromatografie von Phosphatiden, Glykolipiden und Ubichinonen. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 243–253.

28. ANTIBIOTICS

GOSSELINCKX, F. AND PARMENTIER, G.: Chromatographic separation of the components of staphylomycin. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 181–190 — thin-layer, paper and column chromatography.

29. INSECTICIDES AND OTHER PESTICIDES

KOVÁČ, J.: Bestimmung von O,O-Dimethyl-O-(3-methyl-4-nitrophenyl)-thiophosphat in technischen Produkten nach vorhergehender Abtrennung der Begleitstoffe mittels Dünnschichtchromatographie. *J. Chromatog.*, 11 (1963) 412–413 — polarography after chromatography on SiO_2 (Siloxyd).

30. SYNTHETIC AND NATURAL DYES

EGGER, K.: Dünnschicht-Verteilungschromatographie der Plastidenpigmente. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 75–80.

RUIZ, I. SAENZ LASCANO AND LAROCHE, C.: La chromatographie sur couches minces des colorants synthétiques. *Bull. Soc. Chim. France*, (1963) 1594–1597.

32. PHARMACEUTICAL APPLICATIONS

VERCRUYSSE, A.: Chromatography of Doriden (α-phenyl-α-ethyl-glutarimide) in postmortem material on coated glass sheets. *Chromatographie Symposium II, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1962*, publ. 1963, pp. 207–212.

34. RADIOACTIVE COMPOUNDS

SCHULZE, P. E.: Beispiele aus Tritium-Markierung organischer Verbindungen und deren Anwendung in Medizin und Technik. *Angew. Chem.*, 75 (1963) 106 — layer 1 mm thick; gas-flow counter with an ultra-thin diaphragm; 0.3% efficiency for ^3H.

Gas Chromatography

1. REVIEWS AND BOOKS

HOOVER, S. R.: The composition of food flavors. *J. Am. Dietet. Assoc.*, 43 (1963) 19–22 — a review with 18 references.

MILLER, J. M.: *Experimental Gas Chromatography*, Gow-Mac Instruments Co., Madison, N.J., 1963, p. 103 — a laboratory manual for beginners.

NEHER, R.: *Steroid Chromatography*, Elsevier, Amsterdam, 1963 — one of the 7 chapters is concerned with gas chromatography.

STRUPPE, H. G.: IV. Symposium über Gas-Chromatographie in der Deutschen Demokratischen Republik. *Z. Chem.*, 3 (1963) 317–321 — review of the selected papers presented in Leuna, May 1963.

2. FUNDAMENTALS, THEORY AND GENERAL

2c. Thermodynamics and theoretical relationships

GIDDINGS, J. C.: Evidence on the nature of eddy diffusion in gas chromatography from inert (non-sorbing) column data. *Anal. Chem.*, 35 (1963) 1338–1341 — serious differences between experiment and classical theory may be explained by coupling theory $H = 2\lambda d_p + 1(^1/_2)\lambda d_p + 1/C_g'v) + C_g v$.

GORDON, S. M., KRIGE, G. J., HAARHOFF, P. C. AND PRETORIUS, V.: Mobile phase effects in packed liquid chromatography columns. *Anal. Chem.*, 35 (1963) 1537–1539.

KIESELBACH, R.: Gas chromatography: An experimental study of air peaks. *Anal. Chem.*, 35 (1963) 1342–1345 — in HETP-equation, a significant velocity-independent parameter is absent and a mass transfer lag independent of partition coefficient is present.

VAN DEN DOOL, H. AND KRATZ, P. D.: A generalization of the retention index system including linear temperature programmed gas–liquid partition chromatography. *J. Chromatog.*, 11 (1963) 463–471 — Kováts retention index is modified for the case of linear temperature programmed operation and indices of more than 100 compounds on SE-30 and Carbowax 20 M are given.

2d. General

COHEN, E. N., PARZEN, E. AND BAILEY, D. M.: Some observations on linearity of response with the flame ionization detector. *J. Gas Chromatog.*, 1, No. 8 (1963) 14–18 — a logarithmic plot between integrated peak area and concentration of solute (halothane) exists for at least two orders of magnitude above the line of linear response.

STRICKLER, T. D.: Ionization by α-particles in binary gas mixtures. *J. Phys. Chem.*, 67 (1963) 825–830 — usable in evaluation of cross-section detector signal.

3. TECHNIQUES I

3a. Detectors

FLETT, M. S. C. AND HUGHES, J.: The application of a long-path infrared cell in gas chromatography. *J. Chromatog.*, 11 (1963) 434–439 — eluted compound is diverted to the cell and in 4 min the spectrum from 5–15 μ is recorded; less than $3 \cdot 10^{-5}$ g of an impurity could be identified.

HAMPTON, WM. C.: Thermal conductivity bridge circuit. *U.S. Pat.*, 3,091,957, June 4, 1963 — a thermistor type.

NERHEIM, A. G.: Gas density balance employing differential flow measurement. *U.S. Pat.*, 3,091,113, May 28, 1963.

PENN, W. H. AND LOTZ, F. G.: Gas analyzer based on the principle of thermal conductivity. *U.S. Pat.*, 3,088,310, May 7, 1963 — made from glass or quartz surrounded by very tightly packed aluminium powder.

SEIYAMA, T., FUJISHI, K., NAGATANI, M. AND KATO, A.: New detector for gaseous components using zinc oxide thin films. *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 652–655 (English summary p. A 44).

3b. Column performance and filling studies

BÜHRING, H.: Der Einfluss des Trägermaterials auf die quantitative gaschromatographische Analyse höherer Fettsäuremethylester. *J. Chromatog.*, 11 (1963) 452–458 — untreated support materials adsorb considerable amounts of fatty acid esters (measured with ^{14}C labelled compounds) even though symmetrical peaks are obtained.

FOLLAIN, G.: Chromatographie gazeuse. Influence de la nature et de la quantité de soluté sur les temps de rétention. *Rev. Inst. Franc. Pétrole Ann. Combust. Liquides*, 18 (1963) 66–71.

JENTZSCH, D. AND HÖVERMANN, W.: The application of Golay columns of larger internal diameter. *J. Chromatog.*, 11 (1963) 440–451 — good results are obtainable with carrier gas velocities of 10–25 ml/min and a ratio of stationary phase thickness to column diameter of $\sim 10^{-3}$.

VANDENHEUVEL, F. A.: Estimation and correction of post-column dead volume effect in chromatography. *Anal. Chem.*, 35 (1963) 1193–1198 — a study of peak overlapping and distorting by dead volumes.

VAN DER STRICHT, M. AND VAN RYSSELBERGE, J.: Modified Bentone 34, a new addition to stationary phases in gas chromatography to assist in separation of difficult aromatic mixtures. *J. Gas Chromatog.*, 1, No. 8 (1963) 29–33 — Bentone 34 with Apiezon L separates dimethylnaphthalenes at 171°; separation of *o*-, *m*- and *p*-dimethoxybenzenes and chloroacetophenones.

ZLATKIS, A. AND WALKER, J. Q.: Surface modification of capillary columns for use in gas chromatography. *Anal. Chem.*, 35 (1963) 1359–1362 — modifications of Cu-capillaries by means of Ag, Au, Pb or Hg, $K_2Cr_2O_7$, NaCl, silica and epoxy resins.

3c. Apparatus, accessories and materials for GC

ARNETT, E. M. AND DUGGLEBY, P. McC.: A rapid and simple method of deuterium determination. *Anal. Chem.*, 35 (1963) 1420–1424 — a procedure for generating HD–H_2 from mixed isotopic water samples for D_2 determination by thermal conductivity measured by standard GC apparatus.

BURK, M. C.: Chromatographic analyzer peak reader. *U.S. Pat.*, 3,094,862, June 25, 1963.

DANIELS, N. W. R.: Multiple temperature gradient trap for preparative gas chromatography. *Chem. Ind. (London)*, (1963) 1078–1079 — all glass-trap with several consecutive hot and cold zones increased the trapping efficiency.

GODIN, G. W.: Fluid for soap-film flow-meters. *Chem. Ind. (London)*, (1963) 778–778 — silicone fluid MS 200/20cs (Midland Silicone Ltd., Gr. Brit.) 20% by vol. tetradecane forms films as an aqueous soap solution; useful for anhydrous gas streams.

STEELE, E. L.: Analytical instrumentation in space exploration. *Anal. Chem.*, 35, No. 9 (1963) 28A–38A — a GC apparatus for measuring of volatile constituent in lunar crust material; 3 columns (molecular sieve 5A, Carbowax 20 M on Teflon 6, and Apiezon L on H_3PO_4-treated Chromosorb) at 105°.

TARAMASSO, M. AND GUERRA, A.: Microsyringe for gas chromatography. *U.S. Pat.*, 3,094,155, June 18, 1963.

WEBER, O.: Ein automatisches Planimeter zur Auswertung von Streifenblatt-Chromatogrammen (optisches Nachlaufsystem). *Z. Anal. Chem.*, 194 (1963) 334–340 — standard deviation $< 0.4\%$; construction details are described.

WHISMAN, M. L. AND ECCLESTON, B. H.: Purification of radioactive organic compounds: A non-contaminating gas liquid chromatographic system. *Anal. Chem.*, 35 (1963) 1333–1334 — a construction with flame ionization detector.

YOSHIKAWA, Y. AND KUGE, Y.: Simple gas reservoir for gas chromatography. *Japan Analyst*, 12 (1963) 487–487.

4. TECHNIQUES II

4a. Preparative-scale GC

BAYER, E.: Präparative Gas-Chromatographie. *Chimia (Aarau)*, 17 (1963) 199–206.

4b. Programmed temperature GC

VIGDERGAUZ, M. S. AND GOL'BERT, K. A.: (Gas chromatography with the programming of temperature). *Zavodsk. Lab.*, 29 (1963) 1029–1035 — review of theory.

4d. Special microtechniques

BEROZA, M. AND SARMIENTO, R.: Determination of the carbon skeleton and other structural features of organic compounds by gas chromatography. *Anal. Chem.*, 35 (1963) 1353–1357 — identification of structure from hydrocarbon fragment produced by hydrogenolytic reaction (Pd catalyst) of O- and N-compounds.

EVENS, F. M. AND FASSEL, V. A.: Simultaneous determination of oxygen and nitrogen in steels by a d.c. carbon-arc, gas chromatographic technique. *Anal. Chem.*, 35 (1963) 1444–1448 — 0.003–0.1 wt. % O and N can be measured with relative standard deviation of $\pm$ 2.5–10% in $<$ 5 min.

HALEY, A. J.: An extension of the Nelsen-Eggertsen continuous flow method of surface area measurement. *J. Appl. Chem. (London)*, 13 (1963) 329–399 — the size distribution of pores in the 10–300 Å range with $\pm$ 2.5% variation in the range 40–1250 m^2/g is measured by a modification of the N.-E. method; pressures to 150 p.s.i.a. at a fixed rate of 10% N_2 in He are varied.

4e. Automation

MERRITT, J., COMENDANT, F., ABRAMS, S. T. AND SMITH, V. N.: Process gas chromatograph with ultraviolet detector. *Anal. Chem.*, 35 (1963) 1461–1464 — measuring of aromatics at 2357 Å only; linearity for benzene 0–5% and toluene 0–25%

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

KULEY, C. J.: Gas chromatographic analysis of C_1 to C_4 hydrocarbons in the parts per million range in air and in vaporized liquid oxygen. *Anal. Chem.*, 35 (1963) 1472–1475 — detection limits are 0.2–0.08 p.p.m. of C_2–C_4 hydrocarbons for β-ray ionization and 1–10 ml of gas sample and 0.004–0.07 p.p.m. of C_1–C_4 for flame ionization and 10 ml of sample; modified activated Al_2O_3-column.

5b. Other hydrocarbons

ARCHER, E. D. AND FRANCIS, S. A.: Analysis of C_7 olefins by a combination of gas chromatography and nuclear magnetic resonance spectrometry. *Anal. Chem.*, 35 (1963) 1369–1373 — correlation of GC data from 2 columns and of NMR data from GC microfractions.

BECKMAN, H., ALLEN, P. T. AND BERKENKOTTER, P.: Identification of components of the extract of *Chrysanthemum cinerariae* folium separated by gas chromatography. *J. Gas Chromatog.*, 1, No. 8 (1963) 21–22 — on SE-30 by PTGC at 225–300°.

CLEMONS, C. A., LEACH, P. W. AND ALTSHULLER, A. P.: 1,2,3-Tris-(2-cyanoethoxy)-propane as a stationary phase in the gas chromatographic analysis of aromatic hydrocarbons. *Anal. Chem.*, 35 (1963) 1546–1548 — retention data for 30 aromatics and some other compounds at 60°.

FRANCIS, S. A. AND ARCHER, E. D.: Identification of triisobutylene and tetraisobutylene isomers by a combination of gas chromatography and nuclear magnetic resonance techniques. *Anal. Chem.*, 35 (1963) 1363–1367 — 4 trimers and 6 tetramers were identified.

HOIGNÉ, J., WIDMER, H. AND GÄUMANN, T.: Gas-chromatographic identification of phenylalkanes and bicyclic hydrocarbons. *J. Chromatog.*, 11 (1963) 459–462 — Kováts retention indices I and dI/dT for 43 aromatic hydrocarbons derived from benzene, styrene, bicyclohexane, phenylcyclohexane, biphenyl and bibenzyl on Silicone oil DC–710 and Emulphor D at 125–190°.

JOHNSTONE, R. A. W. AND QUAN, P. M.: Naphthalenes in cigarette smoke. *Nature*, 199 (1963) 1184 — 7 naphthalenes (up to trimethyl) were identified.

KNIGHT, J. A., MCDANIEL, R. L. AND SICILIO, F.: Radiolysis products of C_8 and greater carbon content from 2,2,4-trimethylpentane. *J. Phys. Chem.*, 67 (1963) 921–923.

RUSSEL, G. A. AND HENDRY, D. G.: A study of solvent effects in the reactions of methylene. *J. Org. Chem.*, 28 (1963) 1933–1934 — benzene, toluene and cycloheptatriene on squalane and methylsilicone grease.

SCHWARTZ, R. D. AND BRASSEAUX, D. J.: Resolution of complex hydrocarbon mixtures by capillary column gas-liquid chromatography. Composition of the 28° to 114° portion of petroleum. *Anal. Chem.*, 35 (1963) 1374–1382 — mixtures of hexadecane with Kel F-3 and Kel-F 10157 give complete separation of 39 C_5–C_8 hydrocarbons; diode argon microdetector.

WIDMER, H. AND GÄUMANN, T.: Strahlungschemie der Kohlenwasserstoffe. 6. Mitt. Gesättigte Produkte in Hexan. *Helv. Chim. Acta*, 46 (1963) 944–954 — C_3–C_{12} hydrocarbons.

5c. Halogen derivatives of hydrocarbons

NEWMAN, M. S., FRAENKEL, G. AND KIRN, W. N.: The reactions of 4-phenyl-2-butanone and 5-phenyl-2-pentanone with phosphorus pentachloride. *J. Org. Chem.*, 28 (1963) 1851–1853 — chloroolefins on squalane capillary column at 25°.

ODA, N., NORISHIMA, K. AND UCHIJIMA, H.: Determination of α-,β-,γ- and δ-isomers of technical benzene hexachloride by gas chromatography. *Japan Analyst*, 12 (1963) 461–466.

6. ALCOHOLS

BOMBAUGH, K. J. AND THOMASON, W. E.: Gas chromatographic determination of traces of ethanol in methanol. *Anal. Chem.*, 35 (1963) 1452–1454 — 10 p.p.m. on *d*-sorbitol on acetylated Chromosorb at 100°.

BORE, P. AND GATAND, M.: Evaluation quantitative des chaînes grasses saturées dans les tensioactifs non ioniques par chromatographie en phase gazeuse. *Rev. Franc. Corps Gras*, 10 (1963) 399–410 — ethylene oxide derivatives of fatty alcohols can be analysed after HBr treatment and degradation of the molecules to characteristic fragments.

GUVERNATOR, G. C.: Identification of furfuryl alcohol in cigarette smoke. *Tobacco*, 156, No. 14 (1963) 26–26; *Tobacco Sci.*, 7 (1963) 63–63.

9. OXO COMPOUNDS

KATO, R., KIKUCHI, Y. AND USEDA, S. O.: Gas-chromatographic analysis of ethylene glycol *n*-butyl ether in the reaction mixture between ethylene oxide and butan-1-ol. *Japan Analyst*, 12 (1963) 446–450.

NAOKI YOSHIMI, MASAYOSHI YAMAO, MASAYUKI KAWATIKA AND SHIGEYUKI TANAKA: A study on the reaction of formaline with acids by gas chromatography. *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 283–285 — on DEGS at 92°.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

ACKMAN, R. G., BURGHER, R. D. AND JANGAARD, P. M.: Systematic identification of fatty acids in the gas–liquid chromatography of fatty acid methyl esters: A preliminary survey of seal oil. *Can. J. Biochem. Physiol.*, 41 (1963) 1627–1641 — on neopentyl glycol succinate, EGS and SE-30 at 230° and 205°.

FREEMAN, N. K., LINGREN, F. T. AND NICHOLS, A. Y.: The chemistry of serum lipoproteins — in HOLMAN, R. T., LUNDBERG, W. O. AND MALKIN, T. (Editors): *Progress in the Chemistry of Fats and Other Lipids*, Vol. 6, Pergamon Press, Oxford, 1963, pp. 215–250 — apparatus and retention data on pp. 233–238.

HANSEN, R. P.: Occurrence of *trans*-octadec-16-enoic acid in sheep and ox perinephric fats. *Nature*, 198 (1963) 955–995 — on DEGA.

KANESHIRE, T. AND MARR, A. G.: Hydroxy fatty acids of *Azotobacter agilis*. *Biochim. Biophys. Acta*, 70 (1963) 271–277 — retention data of methyl esters of hydroxy fatty acids on DEGS at 187°.

MONDY, N. I.: The effect of storage on the total lipids and the fatty acid composition of potatoes. *J. Agr. Food Chem.*, 11 (1963) 328–329.

OKUI, S., UCHIYAMA, M., MIZUGAKI, M. AND SUGAWARA, A.: Intermediates of the oxidative breakdown of ricinoleic acid by *Candida genus*. *Biochim. Biophys. Acta*, 70 (1963) 346–348 — retention data of methyl esters of hydroxy fatty acid on DEGS at 198°.

PRIVETT, O. S. AND NICKEL, E. C.: Preparation of highly purified fatty acids *via* liquid–liquid partition chromatography. *J. Am. Oil Chemists' Soc.*, 40 (1963) 189–193 — use of GC for purity control of C_{18}–C_{22} acids on DEGS at 175°.

ROUBAL, W. T.: Tuna fatty acids. I. Initial studies on the composition of the light and dark meats of bluefin tuna (*Thunnus thynnus*) — structural isomers of the monoenoic fatty acids. *J. Am. Oil Chemists' Soc.*, 40 (1963) 213–215 — on DEGS at 224°.

ROUBAL, W. T.: Tuna fatty acids. II. Investigations of the composition of raw and processed domestic tuna. *J. Am. Oil Chemists' Soc.*, 40 (1963) 215–218 — on DEGS at 224°, C_{14}–C_{22} fatty acids with up to 6 double bonds.

SCHMELTZ, I., MILLER, R. L. AND STEDMAN, R. L.: Gas chromatographic study of the steam-volatile fatty acids of various tobaccos. *J. Gas Chromatog.*, 1, No. 8 (1963) 27–28 — C_1–C_8 acids on Tween 80 + 2% H_3PO_4.

UCCIANI, E., BUSSON, F. AND CHANET, J.: Étude des huiles d'amandes de *Myrianthus* (Moracées). *Rev. Franc. Corps Gras*, 10 (1963) 393–398 — C_{12}–C_{18} fatty acids on polyester columns at 197° and 225°.

UCHIYAMA, M., SATO, R. AND MIZUGAKI, M.: Characterization of hydroxy acids in depot fat after feeding of ricinoleic acid. *Biochim. Biophys. Acta*, 70 (1963) 344–346 — on DEGS at 200°.

13. STEROIDS

CHAMBERLAIN, J., KNIGHTS, B. A. AND THOMAS, G. H.: Analysis of steroid metabolites by gas chromatography. *J. Endocrinol.*, 26 (1963) 367–387 — retention data of steroids and their acetates, trimethylsilyl ethers and some oxidation products on QF-1, SE-30 and NPGA at 250° and 225°.

FUTTERWEIT, W., MCNIVEN, N. L., NARCUS, L., LANTOS, C., DROSDOWSKY, M. AND DORFMAN, R. I.: Gas chromatographic determination of testosterone in human urine. *Steroids*, 1 (1963) 628–642 — on SE-30 at 240°.

HOLMES, W. L.: Studies on the mechanism of action of cholesterol synthesis inhibitors — in HORNING, E. C. (Editor): *Proceedings, First International Pharmacological Meeting*, Vol. 2, Pergamon Press, Oxford, 1963, pp. 77–84 — sterols and steroids on SE-52 at 228°.

KIRSCHNER, M. A., LIPSETT, M. B. AND WILSON, H.: Metabolism of exogenous dehydroepiandrosterone in man. *Acta Endocrinol.*, 43 (1963) 387–398 — 17-ketosteroids as trimethylsilyl ethers on QF-1 at 187°.

SIMMONDS, P. G. AND LOVELOCK, J. E.: Ionization cross-section detector as a reference standard in quantitative analysis by gas chromatography. *Anal. Chem.*, 35 (1963) 1345–1348 — the quantity of steroid analysis is examined.

15. TERPENE DERIVATIVES

KENNEY, R. L. AND FISHER, G. S.: Reactions on an alkaline Carbowax 20 M column. *J. Gas Chromatog.*, 1, No. 8 (1963) 19–20 — isomerization of 2,8-*p*-menthadien-1-ol was observed.

MITCHEL, B. C., BARBEE, E. P. AND IRBY, Jr., R. M.: The chromatographic determination of menthol in cigarettes and cigarette smoke. *Tobacco*, 156, No. 15 (1963) 30–32; *Tobacco Sci.*, 7 (1963) 64–66.

NAVES, Y. R., ALVES, H. M., ARNDT, V. H., GOTTLIEV, O. R. AND MAGALHAES, M. T.: Études sur les matières végetales volatiles. CLXXXV. Sur les huiles essentielles de deux espèces appartenant au genre *Cryptocaria*. *Helv. Chim. Acta*, 46 (1963) 1056–1059 — linalool, myrcene and 1,8-cineol on Reoplex 100.

VALKANAS, G. AND ICONOMOU, N.: Reaktionen in der Terpen-Reihe. I. Hydratisierung von α-Pinen. *Helv. Chim. Acta*, 46 (1963) 1089–1096 — alcohols and hydrocarbons on 5 stationary phases at 100–162°.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

ICHIRO ISHIZUKA AND MASARU OGATA: Gas chromatographic study of pyridazine-N-oxides and pyridazines. *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 400–401 — 3-methylpyridazine, 1-oxide and 2-oxide of 3- and 4-methylpyridazine at 180°.

LAMPE, F. W., WEINER, E. R. AND JOHNSTON, W. H.: Hydrazine formation in the gas-phase radiolysis of ammonia. *Intern. J. Appl. Radiation Isotopes*, 14 (1963) 231–235 — hydrazine on triethylenepentamine and tetrahydroxyethylethylenediamine at 100°.

YSUKE KOBASHI, HISAKO HOSHAKU AND MIHOKO WATANABE: Pyrolysis of nicotine in air streams. *J. Chem. Soc. Japan, Pure Chem. Sect.*, 84 (1963) 71–74 — 12 nitrogen-containing compounds (pyridines, quinolines, pyrrol *e.g.*) on Carbowax 1500 and 6000 at 140° and 210°.

18. AMINO ACIDS

BLAU, K. AND DARBRE, A.: Preparation of volatile derivatives of amino acids for gas chromatography, *Biochem. J.*, 88, No. 1 (1963) 8P — a review on methods for preparation of volatile derivatives; N-trifluoroacetyl-*n*-amyl esters are preferred.

DARBRE, A. AND BLAU, K.: The quantitative estimation of some amino acids by gas chromatography. *Biochem. J.*, 88, No. 1 (1963) 8P–9P — separated as N-trifluoroacetyl-*n*-amyl esters on PEGA, QF-1 or silicone at 140–180°.

ISHII, S.-I. AND WITKOP, B.: Gramicidin A. I. Determination of composition and amino acid configuration by enzymatic and gas chromatographic methods. *J. Am. Chem. Soc.*, 85 (1963) 1832–1834 — on SE-30 at 175°.

WAGNER, J. AND RAUSCH, G.: Gas-chromatographische Trennung und Bestimmung von Aminosäuren. II. Gas-chromatographische Trennung und Bestimmung von Aminosäuren in Form der Hydroxysäuremethylester. *Z. Anal. Chem.*, 194 (1963) 350–356 — 7 amino acids are converted into hydroxy acids by means of $NaNO_2$ with a reproducibility $< 10\%$ rel.; on silicone oil at 100°.

22. ALKALOIDS

LERNER, M., MILLS, A. L. AND MOUNT, S. F.: Narcotics analysis — a simple approach. *J. Forensic Sci.*, 8 (1963) 126–131 — retention data of 21 alkaloids and related substances on SE-30 at 180–250°.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

GOLDMAN, I. M.: The pyrolysis of pyrazineethanol and 2-pyridineethanol. *J. Org. Chem.*, 28 (1963) 1921–1923 — retention data of vinylpyrazine on DC 550 silicone oil at 190°.

24. ORGANIC SULPHUR COMPOUNDS

CATES, V. E. AND MELOAN, C. E.: Separation of sulfones by gas chromatography. *J. Chromatog.*, 11 (1963) 472–478 — retention volumes of 28 sulfones on Apiezon M and L, Silicone gum rubber and Carbowax 20 M at 200° and 250°, Silicone oil DC 550 at 200° and 225° and Carbowax 1500 at 200°; the best separation was achieved on Carbowax 1500 and 20 M on silanized diatomaceous solid support.

25. ORGANIC PHOSPHORUS COMPOUNDS

DAVIS, A., ROALDI, A., MICHOLOVIC, J. G. AND JOSEPH, H. M.: Applications of gas chromatography to phosphorus containing compounds. *J. Gas Chromatog.*, 1, No. 8 (1963) 23–26 — separations of PCl_3, $POCl_3$ and $PSCl_3$, cyclic $(PNCl_2)_x$ ($x = 3$–7) and dialkyl hydrogen phosphites are described.

26. METALLO-ORGANIC COMPOUNDS

BARRALL, II, E. M. AND BALLINGER, P. R.: Gas chromatographic analysis of lead alkyls with electron affinity detectors. *J. Gas Chromatog.*, No. 8 (1963) 7–13 — retention data on Apiezon L, GE Silicone fluid SF 96 and 1,2,3-tris-(2-cyanoethoxy)-propane at 40–85°; abs. errors ± 3%.

OIWA, T., SATO, M., MIYKAWA, Y. AND MIYAZAKI, I.: Gas-chromatographic analysis of chloromethylsilanes. *J. Chem. Soc. Japan., Ind. Chem. Sect.*, 84 (1963) 409–412 (English summary, p. A29).

29. INSECTICIDES AND OTHER PESTICIDES

GUTENMANN, W. H. AND LISK, D. J.: Rapid determination of 4 (2,4-DB) and a metabolite, 2,4-D, in treated forage by electron affinity spectroscopy. *J. Agr. Food Chem.*, 11 (1963) 304–306 — a herbicide 4(2,4-DB) and a metabolite 2,4-D.

GUTENMANN, W. H. AND LISK, D. J.: The electron affinity detector in pesticide residue analysis. *J. Agr. Food Chem.*, 11 (1963) 301–303 — Cl-pesticide residues at 200°.

31. PLASTICS AND THEIR INTERMEDIATES

NEUMANN, E. W. AND NADEAU, H. G.: Analysis of polyether and polyolefin polymers by gas chromatographic determination of the volatile products resulting from controlled pyrolysis. *Anal. Chem.*, 35 (1963) 1454–1457 — good quantitative results *e.g.* for C_2–C_4 polymers.

32. PHARMACEUTICAL APPLICATIONS

ICONOMOU, N., BÜCHI, J. AND SCHUMACHER, H.: Die Anwendung der Gaschromatographie in der Reinheitsprüfung von Arzneistoffen. Mitt. 8. *Pharm. Acta Helv.*, 38 (1963) 102–118 — purity of low boiling materials used in preparation of drugs and medicines.

ICONOMOU, N., BÜCHI, J. AND SCHUMACHER, H.: Die Anwendung der Gaschromatographie in der Reinheitsprüfung von Drogen und Arzneistoffen. Mitt. 9. *Pharm. Acta Helv.*, 38 (1963) 175–188 — purity of ethers.

KAZYAK, L. AND KNOBLOCK, E. D.: Application of gas chromatography to analytical toxicology. *Anal. Chem.*, 35 (1963) 1448–1452 — retention times of 55 alkaloids, barbiturates and other drugs on SE-30 and QF-1-0065 at 12 different temperatures from 115 to 240°.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

REIN, H. T., MIVILLE, M. E. AND FAINBERG, A. H.: Separation of oxygen and nitrogen by packed column chromatography at room temperature. *Anal. Chem.*, 35 (1963) 1536–1536 — 50 ft. column of 33.5 wt. % DC 200 silicone oil on Chromosorb P at 30° separates O_2 and N_2 (7.3 and 7.6 min) and 7 other gases.

34. RADIOACTIVE COMPOUNDS

CACACE, F., CIPOLLINI, R. AND PEREZ, G.: Continuous elemental analysis of gas chromatographic effluents. Application to the analysis of labeled compounds. *Anal. Chem.*, 35 (1963) 1348–1352 — ratio H:C is measured after conversion to $^{14}CO_2$ and ^3H_2O by means of a flow proportional counter.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

BURDICK, D., SCHMELTZ, I., MILLER, R. L. AND STEDMAN, R. L.: Composition studies on tobacco. XIV. Steam-volatile, neutral substances in various types and grades. *Tobacco*, 156, No. 21 (1963) 30–33; *Tobacco Sci.*, 7 (1963) 97–100.

BURDICK, D. AND STEDMAN, R. L.: Composition studies on tobacco. XV. Steam-volatile, neutral substances in smoke from blended and unblended cigarettes. *Tobacco*, 156, No. 26 (1963) 40–44; *Tobacco Sci.*, 7 (1963) 113–117.

COREY, E. J. AND DAWSON, R. L.: The deamination of *cis*- and *trans*-3-bicyclo-[3,1,0]-hexylamines in aqueous solution. *J. Am. Chem. Soc.*, 85 (1963) 1782–1787 — mentioned hexylamines and hexanols on KOH + Carbowax 20 M and β,β'-oxydipropionitrile at 90°.

ESPOSITO, G. G.: Identification and determination of plasticizers in lacquers by programmed temperature gas chromatography. *Anal. Chem.*, 35 (1963) 1439–1441 — retention data of some phthalates, phosphates and sebacates by means of PTGC to 290°.

FORE, S. P., WARD, T. L., DOLLEAR, F. G.: The preparation of lauryl alcohol and 6-hydroxycaproic acid from petroselinic acid. *J. Am. Oil Chemists' Soc.*, 40 (1963) 30–30 — products of ozonolysis on DEGS at 165°.

JACOBSON, M. AND KOEHLER, H. H.: Components of the flavor of lamb. *J. Agr. Food Chem.*, 11 (1963) 336–339.

KARR, Jr., C., COMBERIATI, J. R. AND WARNER, W. C.: Comparison of pitch resins from different sources by combined pyrolysis and gas liquid chromatography. *Anal. Chem.*, 35 (1963) 1441–1444 — typical differences in C_5–C_8 hydrocarbons and phenols are obtained.

LANSBURY, P. T. AND MACLEAY, R. E.: Temperature dependence of stereochemistry of complex metal hydride reductions. *J. Org. Chem.*, 28 (1963) 1940–1941 — 3,3,5-trimethylcyclohexanone and *cis*- and *trans*-cyclohexanols on Tide detergent by TPGC.

LIBBEY, L. M., BILLS, D. D. AND DAY, E. A.: A technique for the study of lipid-soluble food flavor volatiles. *J. Food Sci.*, 28 (1963) 329–333 — on Apiezon L at 70°.

MATTICK, L. R., ROBINSON, W. B., WEIRS, L. D. AND BARRY, D. L.: Determination of methyl anthranilate in grape juice by electron affinity gas chromatography. *J. Agr. Food Chem.*, 11 (1963) 334–336 — on DC high vacuum grease at 170°.

MCFADDEN, W. H., TERANISHI, R., BLACK, D. R. AND DAY, J. C.: Use of capillary gas chromatography with time-of-flight mass spectrometer. *J. Food Sci.*, 28 (1963) 316–319.

PIPPEN, E. L. AND NONAKA, M.: Gas chromatography of chicken and turkey volatiles: The effect of temperature, oxygen and type of tissue on composition of the volatile fraction. *J. Food Sci.*, 28 (1963) 334–341 — on Tween 20 and Apiezon M at 150°.

SELF, R., CASEY, J. C. AND SWAIN, T.: The low-boiling volatiles of cooked foods. *Chem. Ind. (London)*, (1963) 863–864 — acetone, methanol, sulphides and thiols on Silicone oil and PEG-200 at 25° and 40°.

TRANCHANT, J.: Applications récentes de la chromatographie en phase gazeuse à l'analyse des dérivés halogénés, des amines et des dérivés nitrés. *Bull. Soc. Chim. France*, (1963) 365–367 — 40 references critically reviewed.

WOLFORD, R. W., ATTAWAY, J. A., ALBERDING, G. E. AND ATKINS, C. D.: Analysis of the flavor and aroma constituents of Florida orange juices by gas chromatography. *J. Food Sci.*, 28 (1963) 320–328 — PTGC on Carbowax 20 M and DEGS from 70–245°.

ZULAÏCA, J. AND GUICHON, G.: Séparation et identification de di-esters aliphatiques et aromatiques et de tri-esters phosphoriques par chromatographie en phase gazeuse. *Bull. Soc. Chim. France*, (1963) 1242–1252 — retention data and Kováts indices of symmetrical C_1–C_{10} esters of oxalic, succinic, adipic, suberic, sebacic, phthalic and phosphoric acids on SE-30 and Pneopentyl GS at 200°, 220° and 240°.

ERRATUM

J. Chromatog., 12 (1963) 387

In der Tabelle II im Absatz "stationäre Phase" soll im Falle der Lösungsmittelsysteme S28, S29, S30, S31, S32 und S33 das Wort Formamid ausgelassen werden.

GASCHROMATOGRAPHISCHE RETENTIONSDATEN UND STRUKTUR CHEMISCHER VERBINDUNGEN

I. α-VERZWEIGTE ALIPHATISCHE UND ALICYCLISCHE CARBONSÄURE-METHYLESTER

GERHARD SCHOMBURG

Max-Planck-Institut für Kohlenforschung, Mülheim/Ruhr (Deutschland)

Eingegangen den 2. August 1963)

Die Schwierigkeiten des gaschromatographischen Arbeitens mit analytischer Zielsetzung liegen weniger in der *Trennung* sich strukturell unterscheidender Verbindungen als in deren anschliessender Identifizierung. Die vorliegende Arbeit wird sich mit der *Identifizierung* gaschromatographisch getrennter Verbindungen mit überwiegend gaschromatographischen Mitteln, also über deren Retentionsvolumina in verschiedenen stationären Phasen, befassen. In jeder dieser stationären Phasen ergibt sich für eine bestimmte Verbindung ein nur von der Temperatur und der Polarität dieser Phase abhängiger Verteilungskoeffizient. Trotzdem ist die vollständige Identifizierung komplizierter Mischungen, die etwa Vertreter verschiedener Stoffklassen enthalten, auf gaschromatographischem Wege schwierig. Aus diesem Grunde wird die Kombination gaschromatographischer Trennmethoden mit anderen physikalischen Identifizierungsverfahren oder mit einer chemischen Vortrennung bzw. die Anwendung von Reaktionsgaschromatographie notwendig. Das kann sowohl mit den gaschromatographisch getrennten Komponenten als auch mit der Ausgangsmischung geschehen. Zu den physikalischen Identifizierungsverfahren sind auch die qualitativen oder selektiven Detektoren zu rechnen.

An dieser Stelle sei besonders auf die Kombination gaschromatographischer und massenspektrometrischer Methoden verwiesen[1–7]. Für die ausschliessliche Anwendung von gaschromatographischen Daten zur Identifizierung — besonders von isomeren Verbindungen mit gleicher funktioneller Gruppe — benötigt man ein umfangreiches Vergleichsmaterial, da die Kenntnisse über die Zusammenhänge zwischen Struktur und Retentionsvolumen vorläufig noch beschränkt sind. Für die Unterscheidung von Verbindungen mit verschiedenen funktionellen Gruppen leisten rein gaschromatographische Identifizierungsverfahren wesentliche Hilfe. BAYER[8] verwendet z.B. hierzu seine Selektivitätskoeffizienten, BROWN[9] das "functional groups retention ratio", und auch im KOVATS-schen System[10,11] der Retentionsindices und ΔI-Werte ist eine Identifizierung nach funktionellen Gruppen leicht möglich. Insbesonders das Retentionsindexsystem von KOVATS ist aber auch geeignet, in Substanzklassen mit gleicher funktioneller Gruppe feinere strukturelle Einflüsse zu erfassen. Zur Gewinnung eines umfangreichen Vergleichsmaterials für Strukturaufklärungen mit Hilfe der Gaschromatographie wird entweder eine grössere Zahl von reinen oder annähernd reinen

Substanzen benötigt, oder es sind Mischungen mit Komponenten, deren Zuordnung mit anderen chemischen oder physikalischen Hilfsmitteln leicht möglich ist, erforderlich. Gerade bei Isomeren-Gemischen, die sich nur gaschromatographisch trennen lassen, treten aber die grössten Schwierigkeiten auf, da bei guten Trennungen, wie sie etwa in Kapillarsäulen erreicht werden, die Isolierung von Fraktionen zur Durchführung weiterer Identifizierungsoperationen auf Schwierigkeiten stösst.

Schon in den Anfängen der Gaschromatographie wurden graphische Darstellungen der Logarithmen der Retentionsvolumina gegen die C-Zahl von Gliedern homologer Reihen benutzt, um z.B. zu entscheiden, ob es sich bei einer Substanz, über die nicht mehr als die Art der funktionellen Gruppe und die C-Zahl bekannt sind, um einen ganz bestimmten Isomerentyp handelt. Umgekehrt lässt sich aus solchen Diagrammen natürlich auch die C-Zahl und die Art der funktionellen Gruppen ermitteln.

Zusätzlich wurden in der vorliegenden Arbeit folgende Hilfsmethoden und Informationen zur Identifizierung verwandt:

Auskünfte des Chemikers, von dem die Substanzen präpariert wurden, über Zahl und C-Zahl der zu erwartenden Isomeren in einem Gemisch

Modellsubstanzen, die auf synthetischem, chemisch eindeutigen Wege gewonnen wurden.

Siedepunkte und Siedepunktsregeln.

Massenspektrometrische Kontrollen an Modellsubstanzen und -mischungen sowie an gaschromatographisch getrennten Komponenten.

Die unterscheidende Qualität gaschromatographischer Daten zur Charakterisierung von Substanzen hängt in starkem Masse von der Trennleistung der benutzten gaschromatographischen Säule ab. Je höher die effektive Trennleistung einer gaschromatographischen Säule, definiert etwa durch die Zahl der theoretischen Böden oder andere Kenngrössen wie Trennzahlen, vgl. KAISER[12], ist, um so geringer ist die Wahrscheinlichkeit, dass zwei Isomere mit gleicher funktioneller Gruppe nicht unterscheidbare gaschromatographische Kenngrössen haben. Aus diesem Grunde wurden in der vorliegenden Arbeit alle Messungen mit Kapillarsäulen durchgeführt. Wie bereits oben angedeutet, lassen sich Verbindungen mit verschiedener funktioneller Gruppe natürlich leicht auch durch Wahl einer anderen stationären Phase trennen. Es darf allerdings keine Überlagerung mit anderen Verbindungen zustande kommen. Bei solchen identifizierenden Messungen ist es erstrebenswert, dass die Polarität der verwandten stationären Phasen definiert ist. Das kann erreicht werden, indem man sich konsequent auf eine polare stationäre Phase festlegt, wie es etwa KOVATS gemacht hat, oder verschiedene stationäre Phasen verwendet, deren Polaritätsunterschiede sich durch eine Kenngrösse erfassen lassen. Wegen der verschiedenen Wechselwirkungsarten zwischen den gelösten Molekülen und den Molekülen der stationären Phase ist eine einheitliche Definition der Polarität allerdings schwer möglich. Versuche in dieser Richtung sind von ROHRSCHNEIDER[13] und von BROWN[9] unternommen worden. In Anlehnung an das ROHRSCHNEIDER-sche Konzept wurde in der vorliegenden Arbeit die Differenz der KOVATS-schen Retentionsindices von Benzol (I^B) und Cyclohexan (I^C) hierzu benutzt. Die Tabelle I zeigt, dass bei stationären Phasen, die als polar oder stark polar bekannt sind, diese Differenz ansteigt. Die Indexwerte polarer Verbindungen in polaren stationären Phasen werden um so grösser sein, je polarer diese Phasen sind. Im günstigsten Fall sollte sich ein linearer Zusammenhang

zwischen der Indexdifferenz von Benzol und Cyclohexan in verschiedenen stationären Phasen und dem Retentionsindex einer bestimmten Substanz in diesen Phasen ergeben.

Tatsächlich wird dieser lineare Zusammenhang für Olefine und Aromaten auch gefunden, da bei diesen Verbindungen die gleiche Art der Wechselwirkung zwischen gelösten Molekülen und stationärer Phase wie bei Benzol besteht. Inwieweit diese Polaritätsgrösse auch zur Voraussage der Retentionsindices von Verbindungen mit anderer funktioneller Gruppe geeignet ist, sollen weitere Arbeiten, über die an anderer Stelle berichtet werden wird, zeigen. Die Retentionsindices der Carbonsäuremethylester steigen jedenfalls mit der Grösse der $I^B - I^C$-Differenz — wenn auch nicht vollständig linear — an.

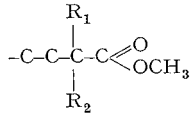
TABELLE I

$I^B - I^C$-WERTE FÜR VERSCHIEDENE STATIONÄRE PHASEN

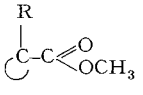
Stationäre Phase	*Temp. (°C)*	$I^B - I^C$	$I^B - I^C + 25$
Squalan	65	−25	0
Polypropylenglykol	60	107	132
Emulphor-O	65	118	143
Polypropylensebazat	65	121	146
Siliconöl XF 1150	65	176	201
Reoplex 400	65	223	248

STRUKTURTYPEN DER UNTERSUCHTEN VERBINDUNGEN

Es wurde eine grössere Zahl von cyclischen und acyclischen Carbonsäuremethylestern mit Alkylverzweigungen in α-Stellung zur Carboxylgruppe gemessen:



R_1 = CH_3 oder H
R_2 = Alkyl oder H

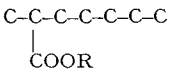


⬡ = 5, 6, 7 oder 8-Ring
R = Alkyl oder H

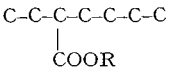
Bei den cyclischen Estern befindet sich die Carboxylgruppe immer direkt am Ring.

Alle Carbonsäureester entstammen der KOCH-schen[14] Carbonsäuresynthese, die im Max-Planck-Institut für Kohlenforschung entwickelt wurde, und wurden mir freundlicherweise von MÖLLER[15] zur Verfügung gestellt, der sich mit Art und Umfang der Isomerisierung unverzweigter Monoolefine bei dieser Synthese befasst hat. In ihrer normalen Ausführungsform werden Olefine in Gegenwart saurer Katalysatoren durch Anlagerung von Kohlenoxyd und Wasser in sekundäre und tertiäre Carbonsäuren umgewandelt. Tertiäre Säuren entstehen dabei durch Methylgruppenwanderung.

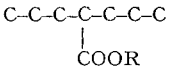
So sind von den Heptancarbonsäuren, die aus Hepten-(1), -(2), -(3) und -(4) entstehen, 5 Isomere zu erwarten:



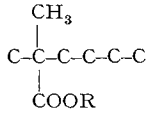
Heptancarbonsäure-(2)-ester



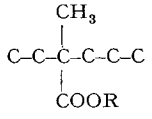
Heptancarbonsäure-(3)-ester



Heptancarbonsäure-(4)-ester



2-Methylhexancarbonsäure-(2)-ester



3-Methylhexancarbonsäure-(3)-ester

Die Zahl der in den jeweiligen C-Zahlbereichen zu erwartenden Isomeren zeigt das Schema:

C-Zahl des Alkans	4	5	6	7	8	9	10	11	12
Isomere mit unverzweigtem Alkan	1	2	2	3	3	4	4	5	5
Isomere mit methylverzweigtem Alkan	1	1	2	2	3	3	4	4	5

Bei Benutzung der richtigen Kontakte werden in Gegenwart von Methanol gleich die Methylester der genannten Carbonsäuren erhalten, die auch zunächst — der geringeren gaschromatographischen Schwierigkeiten wegen — Gegenstand dieser Untersuchungen waren. Es ist abzusehen, dass bei Verwendung geeigneter Zusätze zur stationären Phase auch die freien Carbonsäuren ohne "tailing" mit guter Bödenzahl getrennt werden können.

GASCHROMATOGRAPHISCHE ERGEBNISSE

Für die graphischen Darstellungen des Materials wurden die sog. Nettoretentionsvolumina aus den Chromatogrammen ermittelt und gegen die C-Zahl aufgetragen. Das Gasvolumen der Säule wurde aus dem Retentionsvolumen von Methan, das sich nur wenig von demjenigen von Stickstoff und Sauerstoff unterscheidet, sowie durch Extrapolation aus dem Retentionsvolumen der *n*-Paraffine auf ähnliche Weise wie von EVANS UND SMITH[16] bestimmt.

Fig. 1 zeigt das Chromatogramm der Methylester sämtlicher nach der KOCHschen Synthese möglichen acyclischen Carbonsäuren in den C-Zahlbereichen von C_5–C_{12}, also ohne die Alkancarbonsäure-(1)-methylester. Es wurde mit einer Kapillarsäule mit Polypropylenglykol als stationäre Phase bei einer Bödenzahl von 1000/m aufgenommen. Es handelt sich um das Chromatogramm einer künstlichen oder "master"-Mischung, die allerdings auch praktische Bedeutung hat. Die Zuordnung der einzelnen Peaks zu den verschiedenen Isomeren geschah auf folgende Weise:

1. Von den insgesamt 45 Isomeren im C_5–C_{12}-Bereich standen 9 in Form von Modellsubstanzen zur Verfügung, die auf chemisch eindeutigem Wege dargestellt worden waren.

2. In jedem C-Zahlbereich stand ein Isomerengemisch zur Verfügung, das frei von Verbindungen anderer C-Zahlen war. Werden nur unverzweigte Olefine für die

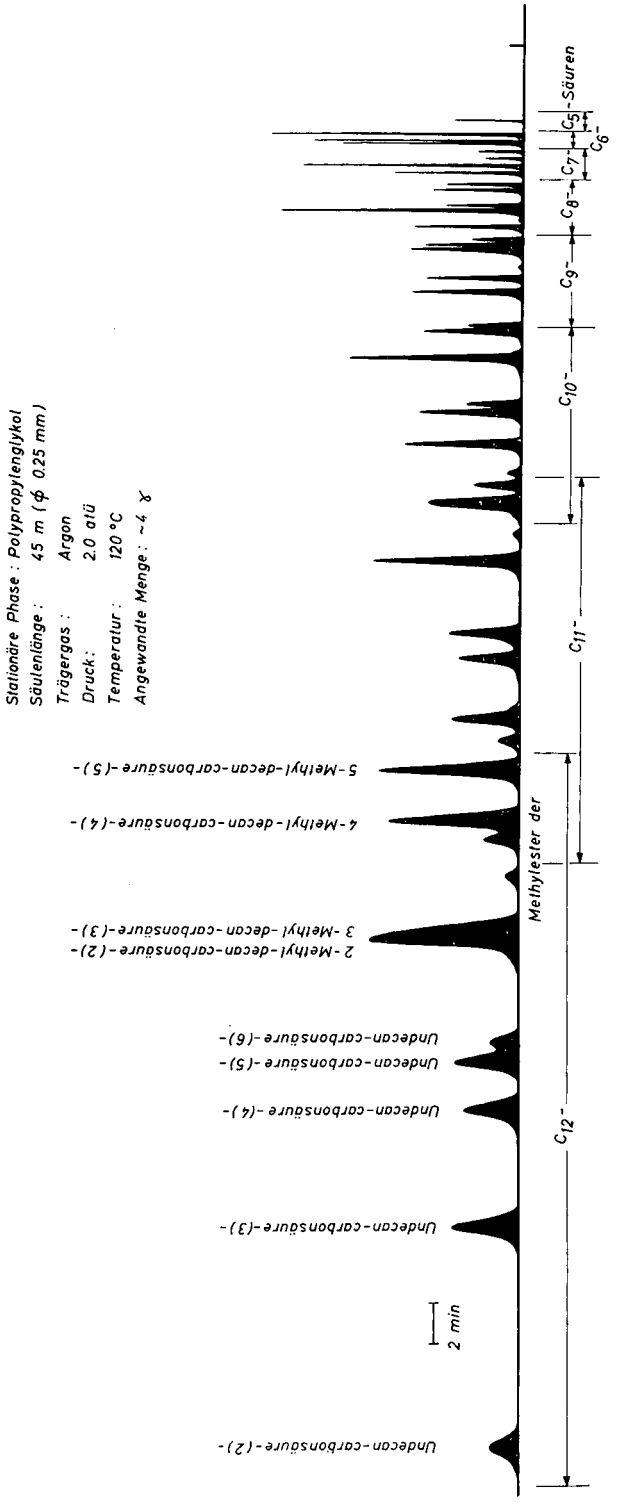


Fig. 1. Methylester der Alkan- und Methylalkancarbonsäuren, die bei der KOCH-schen Synthese entstehen.

KOCH-sche Carbonsäure-Synthese benutzt, so sind weder C-Alkylierungen, die zu Kettenverlängerungen, noch Fragmentierungen, die zur Kettenverkürzung führen, möglich (Kontrolle mit Hilfe der Massenspektrometrie).

3. Auf Grund der von den Siedepunkten solcher und anderer Isomerer bekannten Regeln wurde angenommen, dass das Retentionsvolumen in — zunächst apolaren — stationären Phasen fällt, wenn der Carboxylrest in die Mitte wandert und dass tertiäre Carbonsäureester kleinere Retentionsvolumina haben als sekundäre.

4. Schliesslich wurde die logarithmische Auftragung der Retentionsvolumina gegen die C-Zahl, vgl. Fig. 2, bzw. die KOVATS-schen Indices und die sich auf diese Weise ergebenden Regelmässigkeiten, zur Zuordnung benutzt.

Das Ergebnis der Zuordnung wird zeigen, inwieweit die Annahmen, die insbesondere unter den Punkten 3 und 4 aufgeführt sind, zutreffen und wo sie in bestimmten Fällen zu Irrtümern führen. Aus dem Chromatogramm der Fig. 1 und der graphischen Darstellung der Fig. 2 geht hervor, dass in den C-Zahlbereichen zwischen C_6 und C_9 alle Isomeren der beiden obengenannten Verzweigungstypen getrennt werden

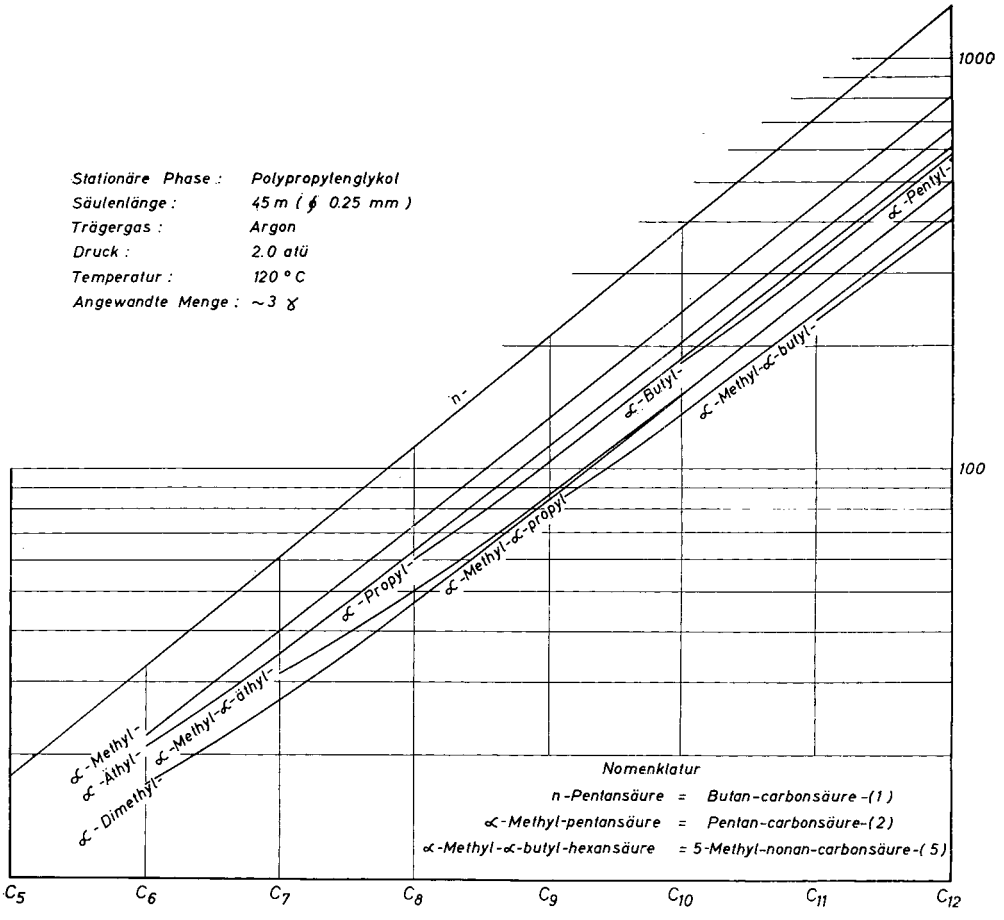


Fig. 2. Retentionsvolumina der am α-C-Atom verzweigten Monocarbonsäuremethylester in Abhängigkeit von der C-Zahl.

können. Von C_{10} an ist es schwierig, die 2-Methylalkancarbonsäure-(2) von der 3-Methylalkancarbonsäure-(3) zu trennen. Im Diagramm der Fig. 2 laufen die Kurven der entsprechenden Homologen von C_{10} an zusammen. Beim Übergang zu einer stationären Phase mit höherer Polarität, etwa Polypropylensebazat, lassen sich die beiden Isomeren noch im C_{11}-Bereich trennen.

Sämtliche Kurven, mit Ausnahme derjenigen der normalen unverzweigten Carbonsäureester, zeigen im gleichen C-Zahlbereich um so stärkere Krümmungen, je verzweigter das Kohlenstoffgerüst ist. Diese Krümmungen können bei der Identifizierung über die Retentionsvolumina besonders bei den niedrigen C-Zahlen zu falschen Zuordnungen führen. Eine eingehende Diskussion der strukturellen Einflüsse auf die Retentionsgrössen soll aber an den Retentionsindices von KOVATS[10,11] erfolgen.

Der Sinn der KOVATS-schen Retentionsindices ist, die für eine Substanz charakteristische gaschromatographische Grösse, das Retentionsvolumen, in eine leicht reproduzierbare Standardform zu bringen, in der sie von den Geräteparametern unabhängig ist. Als Bezugs- oder Standardsubstanzen werden die *n*-Paraffine benutzt. Die KOVATS-schen Indices einer Verbindung gehen ihrer freien Verdampfungsenthalpie aus der betreffenden stationären Phase parallel und sind deswegen zu Betrachtungen über Kohäsion und Wechselwirkung zwischen den Molekülen besonders geeignet. Sie werden an dem willkürlich gewählten Standardmasstab, nämlich dem der freien Verdampfungsenthalpien der *n*-Paraffine, gemessen, die innerhalb eines weiten Temperaturbereichs einen linearen Temperaturgang zeigen. Ausserdem steigen bei den *n*-Paraffinen und anderen homologen Reihen die Siedepunkte bzw. die Verdampfungsenthalpien linear mit der Zahl der Kohlenstoffatome an. Alle anderen Einflüsse auf das Retentionsverhalten rühren bezüglich der gelösten Substanz von der Wechselwirkung der funktionellen Gruppe und den sterischen Faktoren für die Wechselwirkung her. Der Index einer Substanz ist definiert durch die Gleichung:

$$I = 200 \frac{\log V_R^\circ \text{ (Substanz)} - \log V_R^\circ (n\mathrm{P}_z)}{\log V_R^\circ (n\mathrm{P}_{z+2}) - \log V_R^\circ (n\mathrm{P}_z)} + 100 \cdot z$$

wo V_R° = Retentionsvolumen,
nP_z = *n*-Paraffin mit *z* C-Atomen,
z = gerade Zahl.

Die Forderung von KOVATS, dass *z* eine gerade Zahl sein muss, ist nicht berechtigt, da die lineare Abhängigkeit des Retentionsvolumens von der C-Zahl auch für die ungeraden Paraffine erfüllt ist. Der Index hängt ab von der Art der Substanz, der Temperatur und von der Art der stationären Phase. Durch Auswahl mindestens zweier stationärer Phasen, einer apolaren und einer polaren, erhält man ein binäres System von Indexwerten, das zur Charakterisierung von Substanzen nach Molekülgrösse, Verzweigungsgrad des Kohlenstoffgerüstes und Art der funktionellen Gruppen geeignet ist.

Als apolare stationäre Phase wurde Squalan (S), als polare stationäre Phase Polypropylensebazat (PPS), aber auch andere Phasen mit geringerer Polarität benutzt.

Alle Indices wurden mehrfach gemessen, sowohl in Chromatogrammen, die sich über mehrere C-Zahlen erstreckten und somit eine lange Laufzeit hatten wie auch in

TABELLE II

RETENTIONSINDICES DER ALKANCARBONSÄUREMETHYLESTER

Methylester der	*Sdp.* °C	*Squalan*		*Polypropylenglykol*	*Ucon*	*Polypropylensebazat*		ΔI $I^{PPS} - I^{S}$
		120°	*150°*	*120°*	*150°*	*130°*	*150°*	
Pentancarbonsäure-(1)		849		1010		1097		248
Pentancarbonsäure-(2)	138.2	804		945		1027		223
Pentancarbonsäure-(3) *	135.6	796		936		1018		222
2-Me-Butancarbonsäure-(2) *	127.0	763		895		968		205
Cyclopentancarbonsäure		898	902		1080	1190	1196	294
Hexancarbonsäure-(1)		952		1111		1198		246
Hexancarbonsäure-(2) **	160.7	899		1038		1121		222
Hexancarbonsäure-(3) **		884		1020		1100		216
2-Me-Pentancarbonsäure-(2) *, **	147.7	847		974		1048		201
3-Me-Pentancarbonsäure-(3) *, **		869		998		1079		210
Cyclohexancarbonsäure		1007	1011		1186	1300	1309	298
1-Me-Cyclopentancarbonsäure-(1)			925		1084	1181	1184	259
Heptancarbonsäure-(1)		1054		1209		1301		247
Heptancarbonsäure-(2) *		996		1134		1217		221
Heptancarbonsäure-(3)	177.3	976		1110		1190		214
Heptancarbonsäure-(4) *		970		1102		1180		210
2-Me-Hexancarbonsäure-(2) *	168.6	936		1064		1133		197
3-Me-Hexancarbonsäure-(3) *		948		1074		1151		203
Bicycloheptan-(2,2,1)-carbonsäure-(1)			1092		1278	1398	1411	319
1-Me-Cyclohexancarbonsäure-(1)			1026		1183	1275	1285	259
1-Et-Cyclopentancarbonsäure-(1)			1017		1173	1265	1272	255
Octancarbonsäure-(1)		1155		1310		1402		247
Octancarbonsäure-(2)		1094		1232		1316		222
Octancarbonsäure-(3)		1072		1205		1285		213
Octancarbonsäure-(4)		1061		1192		1269		208
2-Me-Heptancarbonsäure-(2)		1030		1156		1225		195
3-Me-Heptancarbonsäure-(3)		1036		1162		1235		199
4-Me-Heptancarbonsäure-(4)		1027		1150		1222		195
1-Et-Cyclohexancarbonsäure-(1)			1122		1278		1383	261
1-Me-Cycloheptancarbonsäure-(1)					1320			
1-Me-Bicycloheptan-(2,2,1)-carbonsäure-(1)			1128		1301		1420	292
Bicyclooctan-(3,3,0)-carbonsäure-(1)			1151		1330		1451	300

Nonancarbonsäure-(1)	1254	1413		1504			250
Nonancarbonsäure-(2)	1192	1331		1414			222
Nonancarbonsäure-(3)	1169	1303		1382			213
Nonancarbonsäure-(4)	1155	1287		1363			208
Nonancarbonsäure-(5)	1150	1282		1357			207
2-Me-Octancarbonsäure-(2)	1128	1254		1323			195
3-Me-Octancarbonsäure-(3)	1128	1254		1327			199
4-Me-Octancarbonsäure-(4)	1112	1236		1306			194
1-*n*-Pr-Cyclohexancarbonsäure-(1)			1200	1353		1455	255
1-Et-Bicycloheptan-(2,2,1)-carbonsäure-(1)				1379			
Decancarbonsäure-(1)		1511		1605			
Decancarbonsäure-(2)		1432		1515			
Decancarbonsäure-(3)		1403		1480			
Decancarbonsäure-(4)		1386		1461			
Decancarbonsäure-(5)		1379		1451			
2-Me-Nonancarbonsäure-(2)		1355		1420			
3-Me-Nonancarbonsäure-(3)		1355		1424			
4-Me-Nonancarbonsäure-(4)		1331		1397			
5-Me-Nonancarbonsäure-(5)		1324		1388			
Undecancarbonsäure-(1)		1613		1705			
Undecancarbonsäure-(2)		1532					
Undecancarbonsäure-(3)		1503					
Undecancarbonsäure-(4)		1485					
Undecancarbonsäure-(5)		1477					
Undecancarbonsäure-(6)		1473					
2-Me-Decancarbonsäure-(2)		1454					
3-Me-Decancarbonsäure-(3)		1454					
4-Me-Decancarbonsäure-(4)		1428					
5-Me-Decancarbonsäure-(5)		1416					

solchen, in denen nur Verbindungen der gleichen C-Zahl vorkamen. Das Chromatogramm der *n*-Paraffine, das zur Bestimmung der Indices benötigt wird, wurde bei wichtigen Messungen sowohl vorher wie hinterher gemessen, um die Langzeitkonstanz der Geräteparameter zu kontrollieren. Bei nicht zu peakreichen Chromatogrammen wurden die *n*-Paraffine den Gemischen direkt zugesetzt. Bei keiner der angewandten Methoden wurden grössere Streuungen als $\pm$ 1 Indexeinheit beobachtet. Naturgemäss sind die Streuungen bei sehr kleinen Retentionsvolumina etwas grösser, wenn bei isothermer Arbeitsweise über einen grösseren C-Zahlbereich hinweg chromatographiert wird.

Die Indexwerte in apolaren stationären Phasen können dazu benutzt werden, um mit Hilfe der Troutonschen Regel die Siedepunkte der betreffenden Verbindungen aus ihnen zu berechnen, vgl. KOVATS[11]. Fig. 3 zeigt den linearen Zusammenhang zwischen den Siedepunkten und den Retentionsindices in der apolaren stationären Phase Squalan für 9 Carbonsäureester, deren Siedepunkte durch MÖLLER[17] nach COTTRELL bestimmt wurden. Ausserdem wurden in der gleichen Figur die Retentionsindices der Alkancarbonsäure-(1)-methylester gegen deren Siedepunkte[18] aufgetragen.

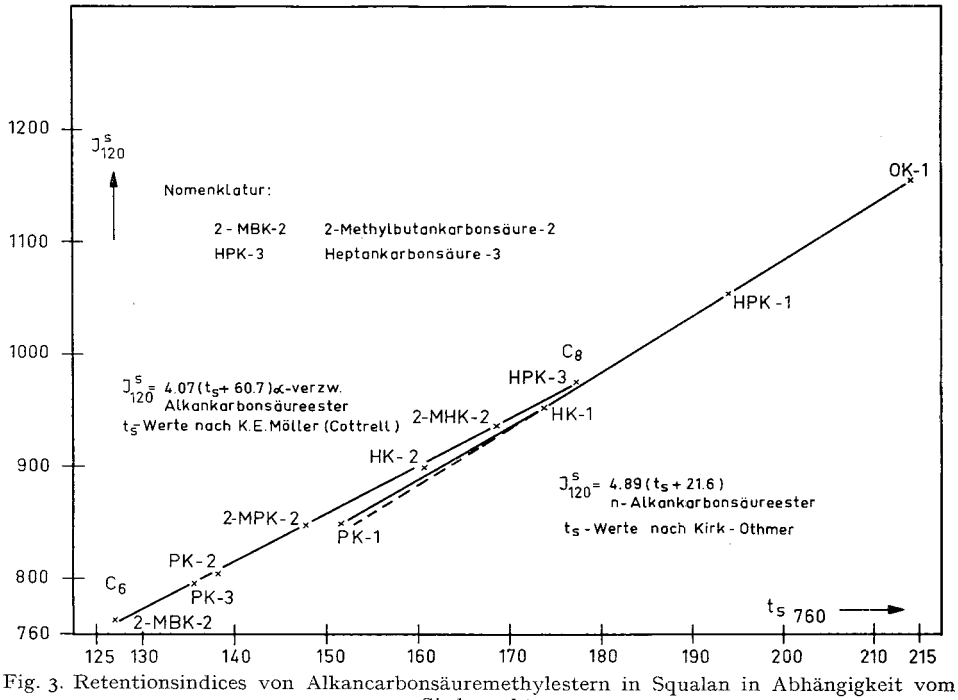


Fig. 3. Retentionsindices von Alkancarbonsäuremethylestern in Squalan in Abhängigkeit vom Siedepunkt.

Die beiden Geraden haben verschiedene Steigungen, sind allerdings auch nicht für die gleichen C-Zahlbereiche gültig. Für den Zusammenhang zwischen Index und Siedepunkt ergeben sich für die beiden Geraden aus dem Diagramm folgende Gleichungen:

$$I^S_{120} = 4.07\ (t_s + 60.7)$$ verzweigte Alkancarbonsäureester
$$I^S_{120} = 4.89\ (t_s + 21.6)$$ Alkancarbonsäure-(1)-ester.

TABELLE III

ZUSAMMENSTELLUNG DER RETENTIONSINDICES GLEICHER ISOMERENTYPEN NACH STEIGENDER C-ZAHL

Methylester der	*Sdp.* °C	*Squalan* 120°	*Polypropylen-glykol* 120°	*Polypropylen-sebazat* 130°	ΔI $I^{PPS} - I^{S}$
Butancarbonsäure-(1)	127.3	760	909	996	
Pentancarbonsäure-(1)	151.5	849	1010	1097	248
Hexancarbonsäure-(1)	173.8	952	1111	1198	246
Heptancarbonsäure-(1)	194	1054	1209	1301	247
Octancarbonsäure-(1)	214	1155	1310	1402	247
Nonancarbonsäure-(1)	224	1254	1413	1504	250
Decancarbonsäure-(1)			1511	1605	
Undecancarbonsäure-(1)			1613	1705	
Pentancarbonsäure-(2)		804	945	1027	223
Hexancarbonsäure-(2)		899	1038	1121	222
Heptancarbonsäure-(2)		996	1134	1217	221
Octancarbonsäure-(2)		1094	1232	1316	222
Nonancarbonsäure-(2)		1192	1331	1414	222
Decancarbonsäure-(2)			1432	1515	
Undecancarbonsäure-(2)			1532		
Pentancarbonsäure-(3)		796	936	1018	222
Hexancarbonsäure-(3)		884	1020	1100	216
Heptancarbonsäure-(3)		976	1110	1190	214
Octancarbonsäure-(3)		1072	1205	1285	213
Nonancarbonsäure-(3)		1169	1303	1382	213
Decancarbonsäure-(3)			1403	1480	
Undecancarbonsäure-(3)			1503		
Heptancarbonsäure-(4)		970	1102	1180	210
Octancarbonsäure-(4)		1061	1192	1269	208
Nonancarbonsäure-(4)		1155	1287	1363	208
Decancarbonsäure-(4)			1386	1461	
Undecancarbonsäure-(4)			1485		
2-Me-Propancarbonsäure-(2)		654	789	854	200
2-Me-Butancarbonsäure-(2)		763	895	968	205
2-Me-Pentancarbonsäure-(2)		847	974	1048	201
2-Me-Hexancarbonsäure-(2)		936	1064	1133	197
2-Me-Heptancarbonsäure-(2)		1030	1156	1225	195
2-Me-Octancarbonsäure-(2)		1128	1254	1323	195
2-Me-Nonancarbonsäure-(2)			1355	1420	
2-Me-Decancarbonsäure-(2)			1454		
3-Me-Pentancarbonsäure-(3)		869	998	1079	210
3-Me-Hexancarbonsäure-(3)		948	1074	1151	203
3-Me-Heptancarbonsäure-(3)		1036	1162	1235	199
3-Me-Octancarbonsäure-(3)		1128	1254	1327	199
3-Me-Nonancarbonsäure-(3)			1355	1420	
3-Me-Decancarbonsäure-(3)			1454		
4-Me-Heptancarbonsäure-(4)		1027	1150	1224	197
4-Me-Octancarbonsäure-(4)		1112	1236	1306	194
4-Me-Nonancarbonsäure-(4)			1331	1397	
4-Me-Decancarbonsäure-(4)			1428		

TABELLE IV

RETENTIONSINDICES DER CYCLOALKANCARBONSÄUREESTER

Methylester der	*Squalan 150°*	*Ucon 150°*	*Sucrose 135°*	*Polypropylensebazat 150°*	*δI/δT in PPS*	*ΔI $I^{PPS} - I^{S}$*
Cyclopentancarbonsäure	902	1080	1105	1196	+0.20	294
1-Me-Cyclopentancarbonsäure-(1)	925	1084	1105	1184	0.15	259
1-Et-Cyclopentancarbonsäure-(1)	1017	1173	1190	1272	0.36	255
Cyclohexancarbonsäure-(1)	1011	1186	1208	1309	0.44	302
1-Me-Cyclohexancarbonsäure-(1)	1026	1183	1198	1285	0.48	259
1-Et-Cyclohexancarbonsäure-(1)	1122	1278	1292	1383	0.60	261
1-*n*-Pr-Cyclohexancarbonsäure-(1)	1200	1353	1368	1455	0.56	255
1-*n*-Bu-Cyclohexancarbonsäure-(1)	1285	1439	1453	1537	0.60	252
1-Me-Cycloheptancarbonsäure-(1)		1320				
Bicycloheptan-(2,2,1)-carbonsäure-(1)	1092	1278	1303	1411	0.64	319
1-Me-Bicycloheptan-(2,2,1)-carbonsäure-(1)	1128	1301	1321	1420	0.64	292
1-Et-Bicycloheptan-(2,2,1)-carbonsäure-(1)		1379				
Bicyclooctan-(3,3,0)-carbonsäure-(1)	1151	1330	1348	1451		300

Die gemessenen Indexwerte der verschiedenen Carbonsäuremethylester in apolaren und polaren stationären Phasen sind in den Tabellen II–IV niedergelegt. Aus diesen Tabellen sind folgende Regeln zu entnehmen:

1. Wandert die Carboxylgruppe durch die unverzweigte Kohlenstoffkette, so nehmen die Retentionsindices ab, je mehr die funktionelle Gruppe in die Mitte wandert.

2. Eine besonders starke Abnahme des Retentionsindex ist beim Übergang von den 1- zu den 2-Carbonsäureestern zu beobachten, während der Unterschied beim Übergang von den 2- zu den 3-, 4- und 5-Alkancarbonsäuren geringer ist.

3. Die Reihenfolge der einzelnen acyclischen Isomeren wird in polaren Säulen auch bei wechselnder Polarität nicht geändert.

4. Bei den methylverzweigten Carbonsäureestern — die Methylgruppe steht immer am gleichen Kohlenstoffatom wie die Carboxylgruppe — hat der 3-Methylalkancarbonsäure-(3)-ester den höheren Retentionsindex als der 2-Methylalkancarbonsäure-(2)-ester. Der 4-Methylalkancarbonsäure-(4)-ester hat wieder einen kleineren Retentionsindex.

5. Die ΔI-Werte (ΔI = Indexdifferenz der gleichen Substanz zwischen der apolaren stationären Phase Squalan und der polaren stationären Phase Polypropylensebazat) sind am grössten für die Alkancarbonsäure-(1)-ester. Sie nehmen ab, je mehr der Carboxylrest in die Mitte der Kohlenstoffkette wandert. Die ΔI-Werte der methylverzweigten Isomeren sind wesentlich niedriger als die der unverzweigten.

6. Bei steigender Kohlenstoffkettenlänge steigen die Retentionsindices um 100 Einheiten pro Kohlenstoffatom, während die ΔI-Werte konstant sind. Zum Teil starke Abweichungen hiervon sind bei den Anfangsgliedern zu beobachten. Sie sind umso grösser, je mehr die Carboxylgruppe in die Mitte wandert. Noch ausgeprägter sind diese Abweichungen bei den methylverzweigten Alkancarbonsäuren.

7. Die cyclischen Carbonsäureester haben höhere Indices und ΔI-Werte als die offenkettigen gleicher C-Zahl, entsprechend den Verhältnissen bei den Kohlenwasserstoffen.

8. Bei Mischungen isomerer Carbonsäureester mit grösserem C-Zahlbereich gibt es bei Abwesenheit von Alkancarbonsäure-(1)-estern C-Zahl-Überschneidungen erst zwischen dem C_{10}- und dem C_{11}-Bereich.

Die bei der Verdampfung eines Stoffes umgesetzte Verdampfungsenthalpie bzw. die bei der Lösung oder Verdünnung einer Verbindung in einer anderen flüssigen Phase auftretenden Lösungsenthalpien sind ein Mass für die zwischen den Molekülen wirkenden Kohäsionskräfte. Zwischen der Lösungsenthalpie ΔH_m und dem Retentionsvolumen besteht ein direkter Zusammenhang über:

$$V_R = V_G + \frac{N_L \cdot RT}{\gamma^\infty \cdot p^0} \cdot V_L$$

worin V_R = Gesamtretentionsvolumen,
V_G = Gasvolumen der Säule,
V_L = Flüssigkeitsvolumen der Säule,
γ^∞ = Aktivitätskoeffizient bei unendlicher Verdünnung des gelösten Stoffes,
N_L = Mole stationärer Flüssigkeit pro Volumeneinheit,
p^0 = Dampfdruck der reinen Substanz (gelöster Stoff),

und

$$\ln p^0 = -\frac{\Delta H_v}{RT} + \text{const.} \quad \text{sowie} \ln \gamma^\infty = \frac{\Delta H_m}{RT}$$

worin ΔH_v = Verdampfungsenthalpie,
ΔH_m = Mischungs- oder Lösungsenthalpie.

Über die Retentionsdaten werden somit Untersuchungen zur intermolekularen Wechselwirkung zwischen stationärer Phase und gelöster Substanz möglich. Die hierbei auftretenden Kräfte können sehr verschiedener Art sein. In der vorliegenden Arbeit ist es überwiegend die Dipol–Dipol-Wechselwirkung und der Einfluss der Abschirmung auf diese, die zusammen mit der immer vorhandenen Dispersionswechselwirkung die Flüchtigkeit der Carbonsäureester über der jeweiligen stationären Phase bestimmt. Abschirmung liegt vor, wenn die Wahrscheinlichkeit vermindert wird, dass die Dipole in günstige Lage zueinander kommen, vgl. EISTERT[20].

In apolaren stationären Phasen erfolgt die gaschromatographische Trennung von apolaren wie von polaren Substanzen entsprechend ihren Siedepunkten, so dass die auftretenden Lösungsenthalpien zumindest proportional den Verdampfungsenthalpien sind. In polaren stationären Phasen kommen die Dipolwechselwirkungskräfte hinzu, wenn die gelöste Verbindung eine funktionelle Gruppe enthält. Die Interpretation gaschromatographischer Daten, die an apolaren stationären Phasen gewonnen wurden, führt zu Ergebnissen, die den durch Vergleich von Siedepunkten gewonnenen äquivalent sind, vgl. HÜCKEL[21]. Das Studium der in polaren stationären Phasen zusätzlich auftretenden Dipolwechselwirkung, die ausserdem starken sterischen Einflüssen unterliegt, ist in dem KOVATS-schen System an den ΔI-Werten möglich, vgl. 5. Die Grösse der ΔI-Werte hängt natürlich auch von der Intensität der Wechselwirkung zwischen stationärer Phase und gelöster Substanz ab, so dass etwas über die Polarität der stationären Phase ausgesagt werden muss, wenn die ΔI-Werte zur Charakterisierung von Substanzen benutzt werden sollen. Für die Diskussion der ΔI-Werte wird die stark vereinfachende Annahme getroffen, dass die

Dispersionskräfte einer Substanz in der apolaren wie in der polaren stationären Phase gleich sind. Der Anteil der Dispersionsenergie an der gesamten Kohäsionsenergie wird auf diese Weise aus den ΔI-Werten eliminiert. Bei der Betrachtung der Indexwerte selbst ist bei polaren stationären Phasen immer mit der Überlagerung beider Effekte zu rechnen. Entsprechend den vorstehenden theoretischen Überlegungen lässt sich zu dem in den obengenannten 8 Regeln zusammengefassten Eigenheiten der gaschromatographischen Daten folgendes sagen:

Zu 1. Die Abnahme des Siedespunktes bzw. des Retentionsindexes in der apolaren Säule mit der Wanderung des Carboxylrestes in die Mitte des Moleküls ist auf die Wirkung des Dispersionseffektes zurückzuführen. Auch die verzweigten Kohlenwasserstoffe, mit dem entsprechenden Kohlenstoffgerüst, deren Kohäsion nur auf Dispersionskräfte zurückzuführen ist, zeigen eine ähnliche Abstufung der Flüchtigkeit (Tabelle V).

TABELLE V

RETENTIONSINDICES DER PENTANCARBONSÄUREESTER UND HEXANE

Methylester	I^S_{120}		I^S_{65}
Pentancarbonsäure-(1)	849	*n*-Hexan	600
Pentancarbonsäure-(2)	804	2-Me-Pentan*	570
Pentancarbonsäure-(3)	796	3-Me-Pentan	585

* Das 2-Me-Pentan hat das gleiche Kohlenstoffgerüst wie die Pentancarbonsäure-(2), wenn das Kohlenstoffatom der Carboxylgruppe mit zum betrachteten Kohlenstoffgerüst gerechnet wird.

Die Umkehrung bei den Retentionsindices von 2-Me-Pentan und 3-Me-Pentan gegenüber den entsprechenden Carbonsäureestern deutet darauf hin, dass bei den letzteren ein Einfluss der funktionellen Gruppe $-COOCH_3$ auf die Dispersionswechselwirkung anzunehmen ist, der von dem einer Methylgruppe abweicht. Die Dipolkräfte, die in polaren flüssigen Phasen zu den Dispersionskräften hinzukommen, unterliegen in starkem Masse der Abschirmung durch die der funktionellen Gruppe benachbarten Alkylgruppen, während bei den Dispersionskräften der Kohlenwasserstoffe durch die Verzweigung ungünstige sterische Verhältnisse für die Wechselwirkung *aller* Atome geschaffen werden. Es gibt keine ausgezeichnete Stelle der Wechselwirkung.

Zu 2. So wird der besonders starke Sprung zwischen den Retentionsindices der Methylester der Pentancarbonsäure-(1) und Pentancarbonsäure-(2) bei der Dipolwechselwirkung durch die Abschirmung der Methylgruppe, die an die Stelle eines Wasserstoffatoms am α-C-Atom getreten ist, hervorgerufen; beim Dispersionseffekt erstreckt sich die Wirkung einer Verzweigungsstelle auf die Wechselwirkung aller Atome des Moleküls. Ein direktes Mass für die Abschirmung der funktionellen Gruppe durch die benachbarten Alkylgruppen kann in der Abhängigkeit der ΔI-Werte im System Squalan–Polypropylensebazat von der Art der abschirmenden Alkylgruppen gefunden werden, vgl. auch 5. So findet man für alle Alkancarbonsäuren-(1) einen ΔI-Wert von etwa 248, für alle Alkancarbonsäuren-(2) von 222. Es ist leicht einzusehen, dass höhere ΔI-Werte geringere Abschirmung der funktionellen Gruppe bedeuten. Die Differenz von 25 Einheiten im ΔI-Wert ist auf den dargelegten Methylgruppeneffekt zurückzuführen. Natürlich spielt auch die Länge des anderen Alkyl-

restes dabei eine Rolle, aber nur unterhalb einer Kettenlänge von C_4. Die Abschirmung einer Alkylgruppe als Inkrement des ΔI-Wertes wird daher aus den ΔI-Werten mit möglichst langem Alkylrest abgeleitet. Bei den Alkancarbonsäuren-(3) werden die ΔI-Werte etwa von der Octancarbonsäure-(3) an, in der neben die Äthylgruppe eine Pentylgruppe am α-C-Atom steht, konstant. Auf diese Weise wurden die in Tabelle VI angeführte Abschirmungsinkremente für die ΔI-Werte ermittelt.

TABELLE VI

ABSCHIRMUNGSINKREMENTE FÜR DIE ΔI-WERTE

(vgl. hierzu Tabelle III)

Alkylgruppe	*Inkrement**
CH_3	25
C_2H_5	34
n-C_3H_7	40
n-C_4H_9	41

* Abnahme des ΔI-Wertes beim Ersatz eines Wasserstoffatoms durch die betreffende Alkylgruppe, wenn der andere Alkylrest vom α-C-Atom länger als n-Pentyl ist.

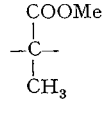
Betrachtet man dagegen die Abnahme der Indices selbst und nicht der ΔI-Werte beim Übergang von der Alkancarbonsäure-(1) zur Alkancarbonsäure-(2), so erhält man erst bei der C_9-Säure eine konstante Verminderung während die ΔI-Werte in allen 5 C-Zahlbereichen konstant sind.

Übergang der IA_{120}-Werte bei	C_6	C_7	C_8	C_9	C_{10}
Wanderung des $-COOCH_3$-Restes aus der 1- in die 2-Stellung	45	53	58	61	62

Wahrscheinlich ist dies auf eine mit der Kettenlänge durch den Einfluss der Kettenverzweigung nicht linear steigende Dispersionsenergie zurückzuführen.

Zu 3. Eine andere Reihenfolge der isomeren Carbonsäureester ist in stärker polaren Säulen nur zu erwarten, wenn ein anderer Typ intermolekularer Wechselwirkung vorliegt oder besonders sterische Verhältnisse bei den Molekülen der stationären Phase einen Einfluss haben. Bei den verwandten stationären Phasen liegen Dipol–Dipol- und Dispersionswechselwirkung in der gleichen Grössenordnung, so dass eine Überkompensation des Dispersionseffektes durch Dipol–Dipol-Wechsel wirkung unwahrscheinlich ist. Ausserdem wirken beide Effekte in der gleichen Richtung.

Zu 4. Kommt durch eine Verzweigung der Kohlenstoffkette eine Methylgruppe am α-C-Atom hinzu, so ist gegenüber den Alkancarbonsäuren-(2) einerseits mit einer weiteren Verminderung der Dispersionskräfte andererseits mit einer Verstärkung der Abschirmung bei der Dipolwechselwirkung zu rechnen. Wandert die Gruppierung



durch die Kohlenstoffkette, so nehmen die ΔI-Werte nicht weiter ab, da die funktionelle Gruppe durch die benachbarte Methylgruppe schon abgeschirmt ist und

für die Dispersionswechselwirkung die Störung, die durch die Methylgruppe hervorgerufen wird, nicht wesentlich vergrössert wird. Der Einfluss der Stellung dieser Gruppierung auf den Dispersionseffekt ändert sich nur wenig. Trotzdem haben die mittelständigen Isomeren niedrigere Indices.

Zu 5. Eine Gegenüberstellung der ΔI-Werte der unverzweigten und methylverzweigten C_9-Carbonsäureester zeigt den Einfluss der Wanderung der Carboxylgruppe und der Methylverzweigung auf die Abschirmungsverhältnisse (Tabelle VII).

Grosse ΔI-Werte bedeuten starke Wechselwirkung zwischen funktioneller Gruppe und stationärer Phase bzw. geringe Abschirmung der ersteren. Die schwächere Abschirmung der 3-Methylalkancarbonsäure-(3) gegenüber der 2- und der 4-Methyl-

TABELLE VII

ΔI-WERTE DER C_9-CARBONSÄUREESTER

Methylester der	*ΔI*	*Methylester der*	*ΔI*
Octancarbonsäure-(1)	247		
Octancarbonsäure-(2)	222	2-Me-Heptancarbonsäure-(2)	195
Octancarbonsäure-(3)	213	3-Me-Heptancarbonsäure-(3)	199
Octancarbonsäure-(4)	208	4-Me-Heptancarbonsäure-(4)	195

alkancarbonsäure-(2) bzw. -(4) ist schwer zu verstehen. Vergleicht man nämlich die Summen der Abschirmungsinkremente der in beiden Molekülen vorhandenen Alkylgruppen, so ergibt sich kaum ein Unterschied:

Methyl + *n*-Propyl = 66
2 × Äthyl = 68 für 2-Methylpentancarbonsäure-(2)

Für die Vertauschung der Retentionszeiten der beiden Isomeren sind wahrscheinlich Unterschiede bei der Dispersionswechselwirkung verantwortlich.

Zu 6. Betrachtet man die Abhängigkeit der Retentionsindices einzelner Isomerentypen von der Kettenlänge (ohne dass sich in der Umgebung der funktionellen Gruppe etwas ändern würde), so wird der Einfluss der Abschirmung sowie der sterischen Eigenheiten des Moleküls auf die Retentionsindices noch einmal offenbar. Bei den Alkancarbonsäuren-(1) ändert sich im Bereich von C_6–C_{12} der Index ziemlich genau um die zu erwartenden 100 Einheiten pro C-Zahl. Auch die ΔI-Werte bleiben über den ganzen Bereich hinweg konstant. Bei den Alkancarbonsäuren-(2) wird erst von dem C_8-Isomeren ab eine konstante Indexdifferenz von 98–99 beobachtet, während die ΔI-Werte ziemlich konstant sind. Bei den Alkancarbonsäuren-(3) wird der Wert von 100 erst etwa im C_{10}-Bereich erreicht, vgl. hierzu Tabelle III. Hier zeigen allerdings auch die ΔI-Werte erst bei dem C_9-Isomeren konstante Werte. Ähnliches gilt für die Alkancarbonsäuren-(4). Noch ausgeprägter sind diese Verhältnisse bei den methylverzweigten Isomeren. Wenn also trotz gleicher Stellung der funktionellen Gruppe bzw. gleichartiger C-Kettenverzweigung erst von einer bestimmten Länge des Alkylrestes bzw. der Kohlenstoffkette die Indexdifferenzen von C-Zahl zu C-Zahl konstant werden, so kann das nur daran liegen, dass die strukturellen Einflüsse auf den Dispersionseffekt das ganze Molekül erfassen. In polaren flüssigen Phasen kommt die verschiedenartige Abschirmung der für die Wechselwirkung entscheidenden Dipole durch Alkylreste verschiedener Länge zustande.

TABELLE VIII

RETENTIONSINDICES DER CYCLISCHEN KOHLENWASSERSTOFFE

	Squalan 150°	*δI/δT*	*PPS 150°*	*ΔI*	*$I_e - I_n$* (Squalan)*
Cyclopentan	599	+0.55	633	34	99
Cyclohexan	688	+0.43	736	48	88
Cycloheptan	824	+0.45	889	65	124
Cyclooctan	946	+0.50	1029	83	146
Bicycloheptan-(2,2,1)	777	+0.45	851	74	77
Bicyclooctan-(3,3,0)	891	+0.50	970	79	191

* Indexdifferenz für den Übergang vom cyclischen zum acyclischen Kohlenwasserstoff in der stationären Phase Squalan.

Die *ΔI*-Werte ändern sich mit steigender Kettenlänge nicht so stark wie die Indexdifferenzen von C-Zahl zu C-Zahl, weil in den *ΔI*-Werten nur die Dipol–Dipol-Wechselwirkung erfasst wird, die von Propyl ab unabhängig von der Kettenlänge ist, da sie im Molekül lokalisiert und an ihr nur die funktionelle Gruppe selbst beteiligt ist.

Retentionsindices der cyclischen Carbonsäureester

Zu 7. Schon die cyclischen Kohlenwasserstoffe haben einen höheren Retentionsindex als die *n*-Paraffine. Ausserdem erhöht sich deren Retentionsindex beim Übergang von der apolaren zur polaren stationären Phase (Tabelle VIII, Hier gilt also die Annahme nicht, dass die Dispersionsenergie in beiden Phasen gleich ist.

Die *ΔI*-Werte der cyclischen Carbonsäureester sollten aus diesem Grunde ebenfalls um den entsprechenden Betrag höher als diejenigen der acyclischen Carbonsäureester sein (Tabelle IX).

Der Übergang vom acyclischen zum cyclischen Carbonsäureester (bei gleicher C-Zahl) zeigt, dass die Indexdifferenz wesentlich kleiner ist als bei den entsprechenden Kohlenwasserstoffen: 49 Indexeinheiten für den Übergang von Pentancarbonsäure-(1) zu Cyclopentancarbonsäure und 99 Indexeinheiten für den Übergang von *n*-Pentan zu Cyclopentan.

Das ist wohl darauf zurückzuführen, dass die Wechselwirkung auf Grund des Dispersionseffektes durch die funktionelle Gruppe behindert wird; die Carboxylgruppe steht nämlich an einer Kettenverzweigung. Die *ΔI*-Werte der beiden Ringsysteme zeigen bei den Alkanen und bei den Alkancarbonsäureestern geringere Differenzen: 44 Indexeinheiten für den gleichen Übergang gegen 34.

Die abschirmende Wirkung von Alkylgruppen, die in α-Stellung zur Carboxyl-

TABELLE IX

INDEXDIFFERENZ ZWISCHEN CYCLISCHEN UND ACYCLISCHEN CARBONSÄUREESTER

Methylester der	*Squalan*		*PPS*		*ΔI*	
Pentancarbonsäure-(1)	849		1097		248	
		49		93		44
Cyclopentancarbonsäure	898		1190		292	
Hexancarbonsäure-(1)	952		1198		246	
		55		102		47
Cyclohexancarbonsäure	1007		1300		293	

TABELLE X

ΔI-WERTE DER NICHT ALKYLIERTEN UND α-ALKYLIERTEN CYCLISCHEN ESTER

Methylester der	*ΔI*	*Methylester der*	*ΔI*	*Differenz*
Cyclopentancarbonsäure	294	1-Me-Cyclopentancarbonsäure-(1)	259	35
		1-Et-Cyclopentancarbonsäure-(1)	254	30
		1-*n*-Pr-Cyclopentancarbonsäure-(1)	259	35
Cyclohexancarbonsäure	302	1-Me-Cyclohexancarbonsäure-(1)	259	43
		1-Et-Cyclohexancarbonsäure-(1)	261	41
		1-*n*-Pr-Cyclohexancarbonsäure-(1)	255	47
		1-Bu-Cyclohexancarbonsäure-(1)	252	50
Bicycloheptan-(2,2,1)-carbonsäure	319	1-Me-Bicycloheptancarbonsäure-(1)	292	27
Bicyclooctan-(3,3,0)-carbonsäure	300			

gruppe stehen, ist bei den cyclischen Estern stärker als bei den acyclischen, aber abhängig von den sterischen Verhältnissen.

Dies zeigt ein Vergleich der ΔI-Werte der nicht alkylierten und in α-Stellung alkylierten cyclischen Ester (Tabelle X). Die abschirmende Wirkung der einzelnen Alkylgruppen ist wenig abhängig von der Art der Alkylgruppe. Zusammenfassend sind folgende Einflüsse auf die ΔI-Werte der cyclischen Carbonsäureester festzustellen:

(a) Dipolwechselwirkung der Carboxylgruppe mit der polaren stationären Phase.

(b) Die abschirmende Wirkung von Alkylsubstitution auf diese Wechselwirkung.

(c) Unterschiede der Dispersionsenergien in polaren und apolaren stationären Phasen.

(d) Sterische Einflüsse der verschiedenen Ringtypen auf die Dipolwechselwirkung.

Die Abschirmung ist bei den cyclischen Carbonsäureestern so stark, dass z.B. in stärker polaren stationären Phasen die α-Methylverbindungen niedrigere Retentionsindices haben als die entsprechenden Grundkörper, obwohl sie die höhere C-Zahl besitzen. Die Chromatogramme in Fig. 4 zeigen die auftretenden Peakvertauschungen bei den Cyclopentyl- und Cyclohexylestern beim Übergang von Squalan zu Polypropylensebazat.

Während in Squalan die Reihenfolge der Retentionsindices der C-Zahl entspricht (in Fig. 5 hat die obere Kurve kein Minimum bei C_9), haben die nicht alkylierten cyclischen Ester in stark polaren stationären Phasen wesentlich höhere Indices als die alkylsubstituierten. Das Minimum bei C_9 in Fig. 5 ist um so tiefer, je polarer die stationäre Phase ist, vgl. auch die Chromatogramme in Fig. 4. Bei dem Substanzpaar Bicyclohepten-(2,2,1)-carbonsäure und seinem α-Methylderivat kommt es *nicht* zur Peakvertauschung, wohl aber rückt die unmethylierte Verbindung in der polaren stationären Phase sehr nahe an die Methylverbindung heran. Es kommt hier keine Vertauschung der Reihenfolge zustande wegen der besonderen sterischen Verhältnisse bei diesem Ringsystem. Die Retentionsindices in apolaren stationären Phasen werden ausschliesslich durch den Dispersionseffekt beeinflusst. Die Dispersionswechselwirkung wird aber besonders durch das tertiäre C-Atom und die sterischen Verhältnisse an diesem beeinträchtigt. Das sieht man an den beiden homologen Reihen der Cyclopentyl- und Cyclohexylcarbonsäureester. Die Indexdifferenzen zwischen zwei Homologen mit einer um eine Einheit verschiedenen C-Zahl nähert sich erst bei höheren C-Zahlen dem zu erwartenden Wert von 100 für gleiche Dipol–Dipol-Wechselwirkung bei gleicher Abschirmung (Tabelle XI).

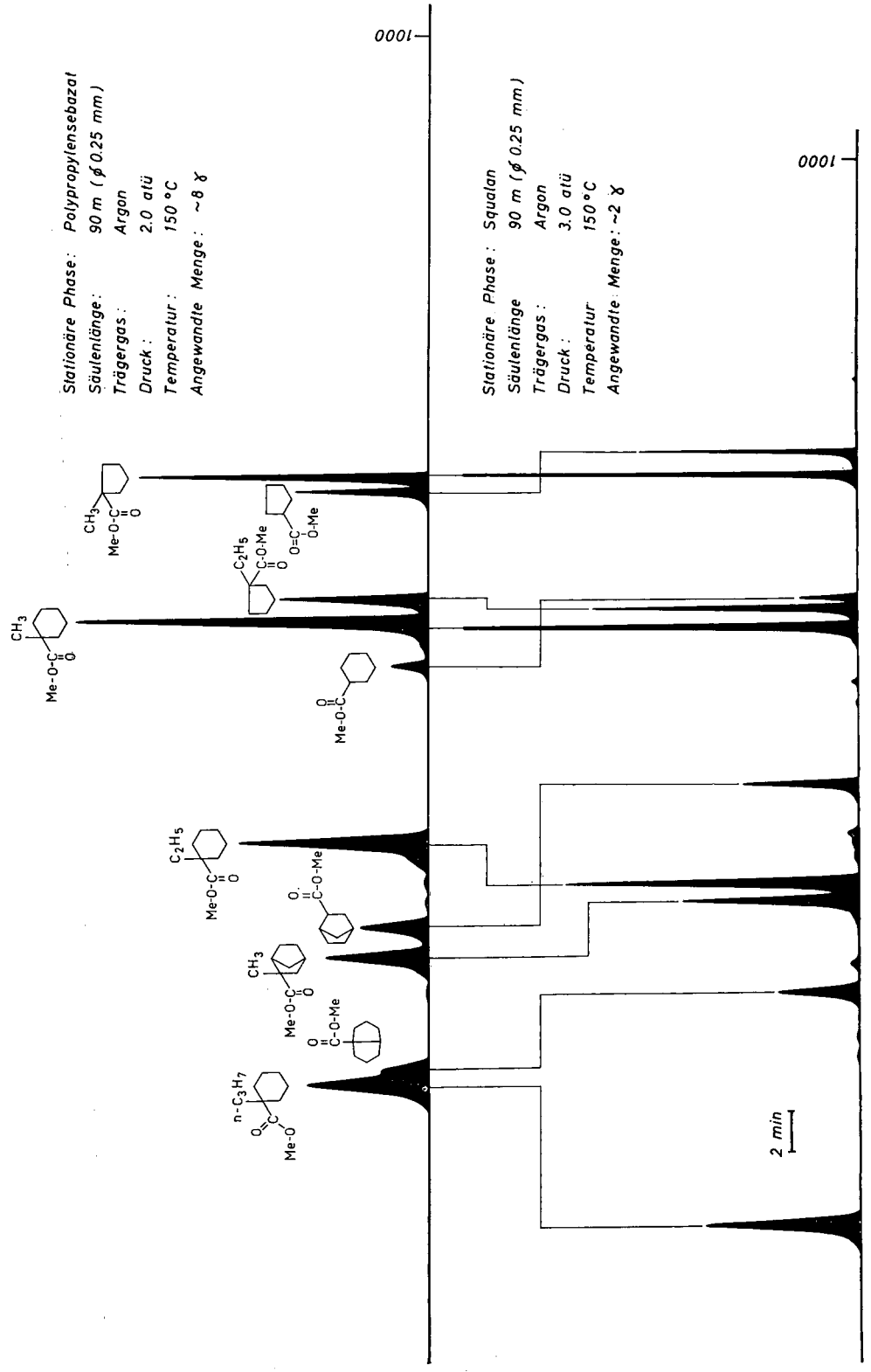


Fig. 4. Einfluss der Alkylsubstitution am α-C-Atom auf die Abschirmung der Carboxylgruppe in Cycloalkancarbonsäuren.

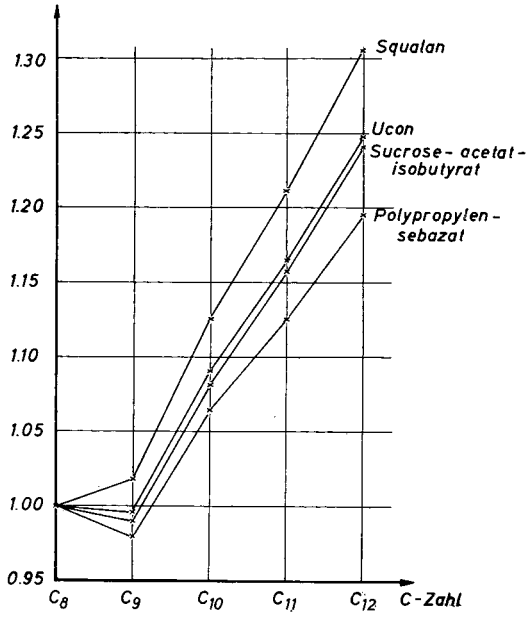


Fig. 5. Relative Retentionsvolumina der Methylester der 1-Alkyl-cyclohexancarbonsäure-(1) in Abhängigkeit von der C-Zahl in vier verschiedenen Säulen.

In stark polaren stationären Phasen kommt es wegen der Dipol–Dipol-Wechselwirkung und deren Abhängigkeit von der Abschirmung durch die in α-Stellung stehenden Alkylgruppen zwar zu den erwähnten Peakvertauschungen, doch ist festzustellen, dass bei diesen stark verzweigten cyclischen Estern die sterischen Einflüsse auf den Dispersionseffekt ebenso wichtig sind wie die Abschirmung der funktionellen Gruppe bei der Dipolwechselwirkung. Ob es gelingt, den Einfluss der Stellung der funktionellen Gruppe in einer Kohlenstoffkette auf den Dispersions-

TABELLE XI

INDEXDIFFERENZ ZWISCHEN HOMOLOGEN CYCLISCHEN CARBONSÄUREESTERN

Methylester der	*Squalan*	*Differenz*
Cyclopentancarbonsäure	902	
		23
1-Me-Cyclopentancarbonsäure-(1)	925	
		92
1-Et-Cyclopentancarbonsäure-(1)	1017	
Cyclohexancarbonsäure	1011	
		15
1-Me-Cyclohexancarbonsäure-(1)	1026	
		96
1-Et-Cyclohexancarbonsäure-(1)	1122	
		78
1-*n*-Pr-Cyclohexancarbonsäure-(1)	1200	
		85
1-Bu-Cyclohexancarbonsäure-(1)	1285	

effekt durch Inkremente von Retentionsdaten zu erfassen, wie es DUNKEL[22] analog für die Verdampfungswärmen bereits versucht hat, wird sich in einer späteren Arbeit zeigen.

DANK

Herrn Prof. Dr. KARL ZIEGLER, dem Direktor des Max-Planck-Instituts für Kohlenforschung, möchte ich für stets gewährte grosszügige Unterstützung und Förderung meiner Arbeit danken.

ZUSAMMENFASSUNG

Es werden die Retentionsdaten für die bei der KOCH-schen Carbonsäuresynthese aus unverzweigten Olefinen entstehenden α-verzweigten cyclischen und acyclischen Carbonsäuremethylester angegeben und die Abhängigkeit dieser Daten—überwiegend in Form der KOVATS-schen Retentionsindices—von der Struktur der zugehörigen Verbindungen diskutiert. Zu den Messungen werden stationäre Phasen verschiedener Polarität in Kapillarsäulen verwandt und der Einfluss der Polarität auf die Trennung der einzelnen Isomerentypen, besonders auch bei den cyclischen Carbonsäureestern, untersucht.

SUMMARY

A detailed investigation of aliphatic and cyclic α-branched carboxylic acids in the C_5–C_{12} range is undertaken by means of capillary column gas chromatography of their methyl esters. The relations between their retention data presented as retention indices (KOVATS) and chemical structure are discussed. The influence of the polarity of the various liquid phases on the separation of isomeric esters is studied. The acids investigated are found in the reaction products of the KOCH synthesis. By the gas chromatographic methods described all components of often complex mixtures of isomeric acids from this process can easily be identified.

LITERATUR

[1] R. S. GOHLKE, *Anal. Chem.*, 31 (1959) 535.
[2] D. HENNEBERG, *Z. Anal. Chem.*, 183 (1961) 12.
[3] L.P. LINDEMANN UND J. J. ANNIS, *Anal. Chem.*, 32 (1960) 1742.
[4] A. A. EBERT, Jr., *Anal. Chem.*, 33 (1961) 1865.
[5] D. HENNEBERG UND G. SCHOMBURG, in M. VAN SWAAY (Herausgeber), *Gas Chromatography 1962*, Butterworths, London, 1963, S. 191.
[6] C. BRUNNÉ, L. JENKEL UND K. KRONENBERGER, *Z. Anal. Chem.*, 189 (1962) 50.
[7] J. A. DORSEY, P. H. HUNT UND M. J. O'NEAL, *Anal. Chem.*, 35 (1963) 511.
[8] E. BAYER, *Angew. Chem.*, 71 (1959) 299.
[9] I. BROWN, *J. Chromatog.*, 10 (1963) 284.
[10] A. WEHRLI UND E. KOVATS, *Helv. Chim. Acta*, 42 (1959) 2709.
[11] E. KOVATS, *Helv. Chim. Acta*, 41 (1958) 1915.
[12] R. KAISER, *Z. Anal. Chem.*, 189 (1962) 1.
[13] L. ROHRSCHNEIDER, *Z. Anal. Chem.*, 170 (1959) 256.
[14] H. KOCH, *Brennstoff-Chem.*, 36 (1955) 321; *Fette, Seifen, Anstrichmittel*, 59 (1957) 493.
[15] K. E. MÖLLER, *Angew. Chem.*, 75 (1963) 1098 (Deutsch), *Intern. Ed.*, 2 (1963) 719 (Englisch).
[16] M. B. EVANS UND J. F. SMITH, *J. Chromatog.*, 6 (1961) 293.
[17] K. E. MÖLLER, Privatmitteilung.
[18] R. E. KIRK UND D. F. OTHMER (Editors), *Encyclopedia of Chemical Technology*, Vol. 5, The Interscience Encyclopedia Inc., New York, 1949, S. 827.
[19] D. HENNEBERG, Privatmitteilung.
[20] B. EISTERT, *Chemismus und Konstitution*, F. Enke Verlag, Stuttgart, 1948, S. 219.
[21] W. HÜCKEL, *Theoretische Grundlagen der Organischen Chemie*, 2. B., 5. Aufl., Akademische Verlagsgesellschaft, Leipzig, 1948, S. 181.
[22] M. DUNKEL, *Z. Phys. Chem.*, 138 (1928) 42.

ZUR CHEMISCHEN KLASSIFIZIERUNG VON PFLANZEN

XXV. QUANTITATIVE BESTIMMUNG VON HASCHISCH-INHALTSSTOFFEN NACH DÜNNSCHICHTCHROMATOGRAPHIE*

FRIEDHELM KORTE UND HELMUT SIEPER

Organisch-Chemisches Institut der Universität Bonn (Deutschland)

(Eingegangen den 24. Juli 1963)

Die Bestimmung der Haschisch-Inhaltsstoffe Cannabidiolsäure (CBDS), Cannabidiol (CBD), Tetrahydrocannabinol (THC) und Cannabinol (CBN) hat beachtliche Bedeutung gewonnen für die polizeiliche Kontrolle des illegalen Haschischhandels[1] und für die Züchtung Haschisch-armer Hanfpflanzen zur Fasergewinnung[2]. In jüngster Zeit gelang es uns, Haschischextrakte an Dimethylformamid-imprägnierten Kieselgel-Dünnschichten in die Cannabinolverbindungen aufzutrennen[3]. Wegen der ausserordentlich grossen Trennschärfe und des geringen Zeitbedarfs schien dieses Verfahren als Grundlage für eine genaue und schnelle Bestimmung der Cannabinole besonders geeignet. Eine solche Methode beansprucht um so grösseres Interesse, als die Literaturmethoden sich im allgemeinen auf eine grobe Bestimmung einzelner Komponenten beschränken[4], oder einen unverhältnismässig grossen Arbeits- und Zeitaufwand erfordern[5].

Die quantitative Bestimmung dünnschichtchromatographisch aufgetrennter Verbindungen erfolgt am besten nach ihrer Elution vom Adsorbens spektrophotometrisch. So konnten wir nach diesem Verfahren genuine Anthrachinonglykoside und -aglykone in Extrakten von *Cortex frangulae* mit einer Messgenauigkeit von $\pm$ 5 % erfassen[6]. Eine analoge Übertragung auf die Cannabisanalytik bereitete jedoch erhebliche Schwierigkeiten. Die direkte spektrophotometrische Auswertung der für die Cannabinole charakteristischen U.V.-Absorptionsmaxima bei 270–280 mμ erwies sich als unmöglich, da wegen der niedrigen Extinktionskoeffizienten dieser Verbindungen für eine genaue Messung nur ungenügend hohe Eluatkonzentrationen erreicht werden konnten. Dagegen gelang es, die getrennten Substanzen auf dem Chromatogramm reproduzierbar in farbige Azoverbindungen mit hohen Extinktionskoeffizienten überzuführen und nach ihrer Elution im sichtbaren Spektralbereich zu photometrieren.

Zum Studium der systematischen Fehler dieser Methode wurde ein Gemisch aus gleichen Teilen von genuinem CBD, synthetischem CBN und synthetischem THC vom Schmp. 62°[7] bandförmig über 16 cm auf Dimethylformamid-gesättigten Kieselgel-Dünnschichten aufgetragen und mit Cyclohexan chromatographiert. Für die Anfärbung der Cannabinole wählten wir Echtblausalz B, Merck, in alkalischer Lösung. Wie ausgeführt wurde[3], entstehen mit diesem Reagens so charakteristisch

* XXIV. Mitteilung: F. KORTE UND H. SIEPER, *J. Chromatog.*, 13 (1964) 90.

gefärbte Verbindungen, dass ihre sichere Identifizierung ohne die Hilfe andersartiger Farbreaktionen an Vergleichschromatogrammen möglich ist. Bei zweimaligem Besprühen wird eine vollständige Umsetzung erreicht, ohne dass sich die schwach gelbe Untergrundfärbung störend auswirken könnte.

Das Ablösen der Farbstoffe vom Adsorbens erfolgte nach der schon für die Anthrachinonbestimmung ausgearbeiteten Elutionstechnik[6]. Als bestes Lösungsmittel eignete sich ein Gemisch aus gleichen Teilen Eisessig und Methanol. Die auf dem Sorptionsmittel irreversibel festgehaltenen Farbstoffmengen waren sehr gering. Die Absorptionsspektren der Farbstofflösungen und eines in analoger Weise erhaltenen Kieselgelblindeluats wurden mit dem Spektrophotometer Beckman DK I in Durchsicht vermessen und sind Fig. 1 zu entnehmen.

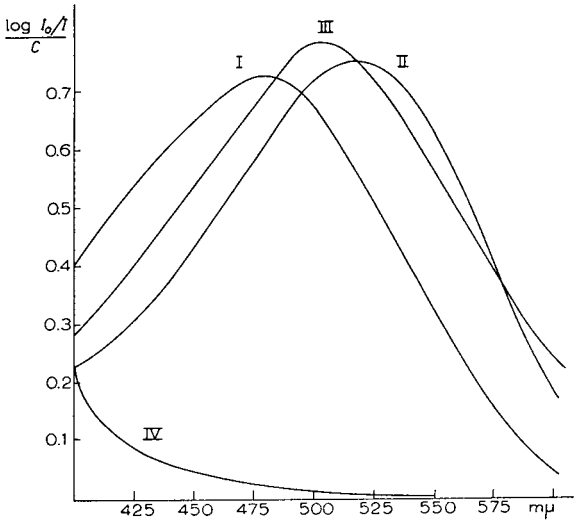


Fig. 1. Absorptionsspektren der Cannabinolverbindungen nach Dünnschichtchromatographie und Umsetzung mit Echtblausalz B (gemessen in Lösung nach Elution m.t Eisessig–Methanol (1:1)). I = CBD-Azofarbstoff; II = CBN-Azofarbstoff; III = THC-Azofarbstoff; IV = Kieselgelblindeluat.

Wie man erkennt, ist die Absorption des Kieselgelblindeluats im Absorptionsmaximum der Cannabinole sehr klein. Die Messergebnisse zeigten die geringste Streuung, wenn die Substanzlösung gegen reines Lösungsmittel vermessen und die getrennt bestimmte Kieselgelblindabsorption davon abgezogen wurde. Die Lösungen zeigten auch nach 3-monatigem Stehen keine Änderung der Absorptionslage und Extinktionshöhe.

Um Aussagen über den Gültigkeitsbereich des Lambert-Beerschen Gesetzes machen zu können, wurden 0.1-proz. Lösungen der Cannabinole in unterschiedlichen Mengen chromatographiert und spektrophotometriert. Die Ergebnisse sind in Fig. 2 dargestellt.

Die maximale Abweichung der Messpunkte der Ausgleichsgeraden liegt zwischen Konzentrationen von 2 und 8 μg/ml bei 5 voneinander unabhängigen Bestimmungen unter $\pm$ 5 %. Innerhalb dieses Konzentrationsbereiches verlaufen die Kurven linear. Daraus kann man schliessen, dass auf dem Chromatogramm eine stöchiometrische

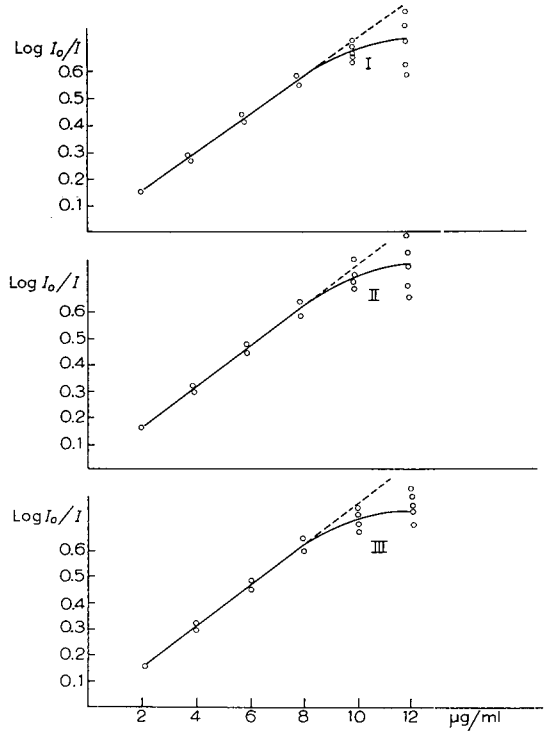


Fig. 2. Eichkurven der Cannabinolverbindungen nach Dünnschichtchromatographie und Umsetzung mit Echtblausalz B (gemessen in Lösung nach Elution mit Eisessig–Methanol (1 : 1) im Maximum der Absorption). I = CBD-Azofarbstoff; II = CBN-Azofarbstoff; III = THC-Azofarbstoff.

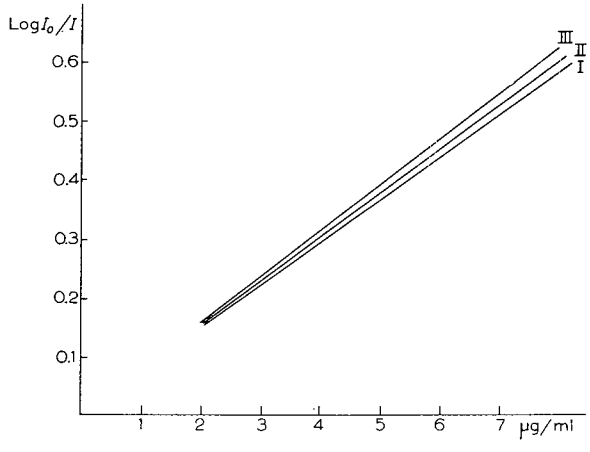


Fig. 3. Eichkurven der Cannabinolverbindungen im Gültigkeitsbereich des Lambert-Beerschen Gesetzes. I = CBD-Azofarbstoff, $\frac{\log I_0/I}{C} = 72.5$; II = CBN-Azofarbstoff, $\frac{\log I_0/I}{C} = 75.0$; III = THC-Azofarbstoff, $\frac{\log I_0/I}{C} = 78.8$.

oder zumindest proportionale Umsetzung der Cannabinole mit dem Reagens und eine der Farbstoffmenge proportionale Elution erfolgt.

In Fig. 3 sind die Ausgleichsgeraden im Linearitätsbereich gesondert aufgezeichnet. Die daraus ermittelten praktischen Extinktionskoeffizienten gestatten die direkte Berechnung unbekannter Cannabinolkonzentrationen nach dem Lambert-Beerschen Gesetz aus den Extinktionswerten der Dünnschichteluate.

Cannabis- oder Haschischextrakte können ohne vorherige Reinigung für die Bestimmung der Cannabinole eingesetzt werden. Farbige Begleitsubstanzen sowie Cannabinol-fremde Substanzen, die mit Echtblausalz farbige Verbindungen geben, stören im allgemeinen nicht, da ihre Laufgeschwindigkeiten auf dem Chromatogramm von denen der Cannabinole genügend verschieden sind. Cannabidiolsäure konnte nach der beschriebenen Methode nicht bestimmt werden, da sie im Lösungsmittelsystem Dimethylformamid–Cyclohexan am Startpunkt zurückbleibt. Versuche, adsorptionschromatographisch an Dünnschichten in einen günstigen R_F-Bereich zu kommen, sind noch nicht abgeschlossen. Wie berichtet wurde[3], waren in allen untersuchten Haschischextrakten drei auf dem Chromatogramm eng benachbarte, aber scharf voneinander getrennte Tetrahydrocannabinole nachweisbar, die mit THC I,

TABELLE I

GEHALT AN CANNABINOLEN IN CANNABISEXTRAKTEN*

	% *der Trockendroge*			
	CBD	*THC I*	*THC II*	*CBN*
Nigeria UNC 59	—	0.079	0.019	0.481
Brasilien UNC 61	—	0.190	Spuren	0.410
Cypern UNC 33	—	0.230	0.150	0.060
Marocco UNC 21	0.129	0.096	Spuren	0.123
Genf UNC 51	0.129	0.059	0.028	0.017
Kanada UNC 37	0.103	Spuren	Spuren	—

* Der Zeitbedarf für die Bestimmung der 4 Komponenten in Rohextrakten liegt bei 4–5 Stunden. Zwei getrennte Analysen reichen im allgemeinen für eine Genauigkeit von ± 5 % aus.

THC II und THC III bezeichnet wurden. THC III war in nicht bestimmbar kleinen Mengen enthalten. Für eine angenäherte Bestimmung von THC I und THC II aus den Extinktionswerten der Umsetzungsprodukte mit Echtblausalz wurde der für das synthetische THC vom Schmp. 62° ermittelte Extinktionskoeffizient eingesetzt (siehe Eichkurve III in Fig. 3). Als Beispiel einer Bestimmung sind die Analysenwerte einiger Haschischextrakte in Tabelle I zusammengestellt.

EXPERIMENTELLER TEIL

Je 10 g Haschisch oder lufttrockener und von groben Stengeln befreiter Cannabis werden in 50 ml dest. Petroläther (Sdp. 40–60°) suspendiert und 2 Min. bei Raumtemperatur unter ständigem Durchleiten eines lebhaften N_2-Stromes mit einem Ultraturrax zerkleinert und extrahiert. Der Filtrationsrückstand wird 3–5 mal mit je 30 ml Petroläther nachextrahiert, bis das Filtrat eine nur noch schwache Farbreaktion mit Echtblausalz B gibt. Die vereinigten Filtrate werden bei Raumtempera-

tur in einem Rotationsverdampfer zur Trockne eingedampft und mit Heptan, dem wenige Tropfen Methyläthylketon und Äthanol zugefügt wurden, auf genau 10-proz. Lösungen eingestellt.

Auf 20 × 20 cm Glasplatten werden Dünnschichten von Kieselgel G, Merck, in einer Dicke von 250 μ mit dem Streichgerät der Fa. C. Desaga, Heidelberg, nach der Vorschrift von STAHL[8] hergestellt und in der früher beschriebenen Weise[3] mit einer Mischung von 60 Vol. % N,N-Dimethylformamid und 40 Vol. % CCl_4 über eine Chromatographiestrecke von 12 cm imprägniert.

Während des Abdunstens von überschüssiger Imprägnierungsflüssigkeit, wozu 1–2 Stunden erforderlich sind, werden 2.5 cm oberhalb der unteren Schichtkante 10–30 μl des zu bestimmenden Haschischextraktes mit einer Mikrosyringe, 3 cm von der Seitenkante her angefangen, über eine Strecke von 13 cm bandförmig aufgetropft. Dabei ist darauf zu achten, dass die Sorptionsschicht nicht beschädigt wird und sich die Testlösung über höchstens 0.5 cm nach oben und unten ausbreitet. Auf dem ausgesparten 3 cm breiten Seitenstreifen können für Vergleichszwecke 3 μl einer 0.1-proz. Lösung eines Gemisches von CBD, CBN und THC oder ein Cannabis- bzw. Haschischextrakt bekannter Inhaltsstoffe aufgetragen werden. Es wird mit Cyclohexan chromatographiert, wozu ca. 30 Min. benötigt werden.

15 mg Echtblausalz B, Merck, das zweckmässigerweise bei 0° aufbewahrt wird, werden in 20 ml N/10 NaOH kalt gelöst und durch eine Glasfritte filtriert. Die Lösung wird beim Stehen allmählich braun und ist nach 5 Min. nicht mehr verwendbar. Sie wird daher unmittelbar nach ihrer Herstellung mit einem Sprüher der Fa. C. Desaga unter N_2-Druck auf die waagerecht liegende Platte im Abstand von ca. 30 cm so aufgenebelt, dass die gesamte Adsorptionsschicht gleichmässig transparent wird, ohne dass bei seitlicher Betrachtung ein Feuchtigkeitsfilm sichtbar wird. Das Chromatogramm wird mit dem Kaltluftstrom eines Föhns getrocknet und wie vorher mit einer frischen Reagenslösung nachbehandelt. CBDS bleibt als orangeroter Fleck am Startpunkt zurück. Es folgen mit steigenden R_F-Werten: CBD (orangerot), CBN (dunkelviolett), THC I (weinrot) THC II (hellviolett) und THC III (rosa).

Die 0.3–1 cm breiten Zonen der Cannabinol-Azofarbstoffe werden mit einem spitzen Bleistift umrandet, mit einem flachen Spatel von 3 mm Breite abgekratzt und quantitativ in Röhrchen von 2 ml Fassungsvermögen übergeführt. Der Farbstoff wird durch Schütteln mit 1 ml eines Gemisches aus gleichen Vol. Teilen Eisessig und Methanol extrahiert. Man lässt absitzen und filtriert mit reduziertem Wasserstrahlvakuum durch eine Glasfritte Schott G 3 unmittelbar in einen 2.5 ml Messkolben. Es wird zweimal mit je 0.5 ml Lösungsmittel nachextrahiert und bis zur Marke aufgefüllt. Zur Herstellung einer Kieselgelblindlösung wird unterhalb der Startlinie des Haschischextraktes eine den Testmengen entsprechende Menge substanzfreier, aber mit der Reagenslösung behandelte Kieselgelzone extrahiert.

Die Absorptionsspektren der Cannabinol-Azofarbstoffe und des Kieselgelblindeluats werden mit einem selbstregistrierenden Spektrophotometer im Wellenlängenbereich von 600–400 mμ gegen reines Lösungsmittel (Eisessig–Methanol (1:1)) aufgenommen. Vom Extinktionswert der Substanzlösung im Wellenlängenmaximum wird der Kieselgelblindwert gleicher Wellenlänge abgezogen. Zur Konzentrationsberechnung nach dem Lambert-Beerschen Gesetz werden folgende Extinktionskoeffizienten eingesetzt: CBD 72.5; CBN 75.0; THC I und THC II 78.8.

DANK

Wir danken der Firma Dr. W. SCHWABE, Karlsruhe, dem Bundeskriminalamt, Wiesbaden, und dem European Office of United Nations Narcotic Drug Section, Genf, für die freundliche Überlassung von Cannabis- und Haschischproben.

ZUSAMMENFASSUNG

Die Haschisch-Komponenten Cannabidiol, Tetrahydrocannabinol und Cannabinol werden nach dünnschichtchromatographischer Trennung und Umsetzung zu Azofarbstoffen spektrophotometrisch bestimmt. Die neue Methode ist auch auf Cannabis- und Haschisch-Rohextrakte anwendbar.

SUMMARY

The hashish components cannabidiol, tetrahydrocannabinol and cannabinol are determined spectrophotometrically after separation by thin-layer chromatography and formation of azo-derivatives. The new method can also be applied to crude extracts of cannabis and hashish.

LITERATUR

1 C. G. FARMILO, *United Nations Secretariat Document*, St/StOA/SER S/7 (1962).
2 C. G. FARMILO, *United Nations Secretariat Document*, St/StOA/SER S/4 (1961) 15.
3 F. KORTE UND H. SIEPER, *J. Chromatog.*, 13 (1964) 90.
4 C. C. FULTON, *Ind. Eng. Chem.*, 14 (1942) 407;
A. RADOŠEVIĆ, M. KUPINIĆ UND LJ. GRLIĆ, *Nature*, 195 (1962) 1007;
LJ. GRLIĆ, *J. Pharm. Pharmacol.*, 13 (1961) 637.
5 O. E. SCHULTZ UND G. HAFFNER, *Arch. Pharm.*, 293 (1960) 1.
6 H. SIEPER, R. LONGO UND F. KORTE, *Arch. Pharm.*, 296 (1963) 403.
7 F. KORTE UND H. SIEPER, *Ann.*, 630 (1960) 71.
8 E. STAHL, *Chemiker-Ztg.*, 82 (1958) 323; *Z. Anal. Chem.*, 181 (1961) 303; *Arch. Pharm.*, 292 (1959) 411.

THE PARTITION OF POLAR AND NON-POLAR LIPIDS IN A REVERSED-PHASE CHROMATOGRAPHIC SYSTEM

A. G. VERESHCHAGIN

K. A. Timiryazev Institute of Plant Physiology, Academy of Sciences of the U.S.S.R., Moscow (U.S.S.R.)

(Received July 25th, 1963)

The additive nature of the chromatographic partition process was postulated by MARTIN already in 1950[1]. He formulated the equation:

$$\ln\left(\frac{\alpha_B}{\alpha_A}\right) = \frac{\Delta\mu_X}{RT} \qquad (1)$$

where A, B are members of a homologous series, differing by the functional group X, α is the partition coefficient and $\Delta\mu_X$ is the difference in chemical potential of the group X in polar and non-polar phases of the chromatographic system.

It follows, that each group in the solute molecule contributes more or less independently to the difference in standard free energy of the solute between the two different phases. Thus, in general, there is a linear relationship between $\ln\alpha$ or $\log_{10}\alpha$ and the number of functional groups in a homologous series. This relationship has been widely used for the chromatographic identification of flavonoids[2], 2,4-dinitrophenylhydrazones of aliphatic aldehydes and methyl-ketones[3], aliphatic and aromatic hydrocarbons[4], and other substances.

In a reversed-phase partition chromatography of lipids[5,6] several attempts were made to derive some information as regards identity from the behaviour of these substances during separation. The R_F value[7] and the logarithm of the retention volume[8] were reported to be linear functions of the number of carbon atoms in an aliphatic chain. In other studies no such linearity was found[9].

It seemed desirable to find the relation between properties of lipid molecules and the chromatographic partition pattern. The following reversed-phase systems were used: for the fatty acids—natural rubber + aliphatic hydrocarbons, b.p. 220–260°/90 % acetic acid; for triglycerides — aliphatic hydrocarbons, b.p. 260–310°/a mixture of acetone and acetic acid (85:15 v/v). The experimental conditions used for the separation and localization of lipids have been described previously[10,11].

The molecular properties of lipids were characterized by the polarity constant:

$$K_{1,2} = 100 - m + 2e \qquad (2)$$

where m is the number of carbon atoms and e the number of double bonds in a lipid molecule.

In a reversed-phase system the absolute R_F values of fatty acids are not readily reproducible owing to slight fluctuations of temperature during the chromatographic

procedure; therefore, the stable relative value R_1 was used instead of R_F for the calculation of the partition coefficient α_1. The R_1 value is computed from the equation:

$$R_1 = \frac{\text{distance travelled by a given fatty acid zone}}{\text{distance travelled by ricinoleic acid zone}} \quad (3)$$

Table I gives the K_1 and R_1 values of higher fatty acids (the abbreviated designations of the acids are given in parentheses).

TABLE I

POLARITY CONSTANTS AND R_1 VALUES OF HIGHER FATTY ACIDS

Fatty acid	*Stearic (S)*	*Palmitic (P)*	*Oleic (O)*	*Linoleic (L)*	*Myristic (M)*	*Linolenic (Le)*	*Lauric (La)*
K_1	82	84	84	86	86	88	88
R_1	0.10	0.18	0.18	0.28	0.34	0.39	0.52

The partition coefficient was calculated according to MARTIN AND SYNGE[12]. For the reversed-phase system at a constant temperature the effect of the water content of the solid support was negligible; therefore, it was assumed that:

$$\frac{q_{\text{polar}}}{q_{\text{non-polar}}} = 1 \quad (4)$$

and:

$$\alpha_{1,2} = \frac{1}{R_{1,2}} - 1 \quad (5)$$

There is a linear relationship between $\log_{10}\alpha_1$ and the polarity constant of saturated fatty acids (Fig. 1). The unsaturated fatty acid with carbon number m and the saturated acid with $m - 2e$ carbon atoms have equal polarity constants, but the $\log_{10}\alpha_1$ values of these pairs differ considerably from each other, with the exception of the pair oleic–palmitic acid. The $\log_{10}\alpha_1$ value is a linear function of the polarity constant of unsaturated fatty acids. Using the curves of Fig. 1, it is possible to determine the values of m and e from the experimental R_1 value of the unknown fatty acid.

The chromatographic behaviour of triglycerides obtained from linseed, poppyseed, and cottonseed oils was characterized by the value R_2 (ratio of R_F of triglyceride to the R_F of butyl hexabromostearate). The triglycerides, together with their polarity constants, K_2, and R_2 values are listed in Table II, columns 1–3. There was a linear relationship between K_2 and the partition coefficient of triglycerides α_2 (Fig. 2).

The data of Table II show that glycerides with different fatty acid compositions may still have the same polarity constants. Triglycerides of the same polarity always form a separate chromatographic zone. Further separation of such mixtures into individual triglycerides was achieved by quantitative bromination of the double bonds by the KAUFMANN reagent[13]. The polarity constant of brominated triglycerides can be expressed by the equation:

$$K_3 = m - s \quad (6)$$

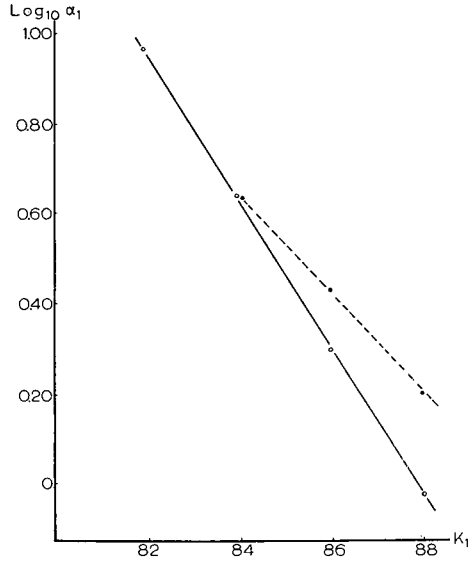


Fig. 1. Dependence of the logarithm of the partition coefficient of fatty acids on the polarity constant K_1. O——O saturated fatty acids; ●- - - - -● unsaturated fatty acids.

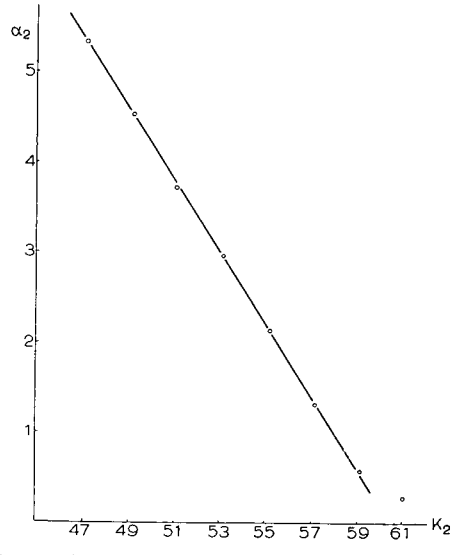


Fig. 2. Dependence of the partition coefficient of higher fatty acid triglycerides on the polarity constant K_2.

TABLE II

POLARITY CONSTANTS AND R_2 VALUES OF TRIGLYCERIDES BEFORE AND AFTER BROMINATION

Triglycerides	K_2	R_2	K_3	R_2'
SPO	47	0.16	—	—
PPO			51	0.21
SPL			53	0.25
POO	49	0.18	54	0.27
SOL			56	0.30
OOO			57	0.34
PPL			51	0.29
POL	51	0.21	54	0.36
SLL			56	0.42
OOL			57	0.47
PLL	53	0.25	54	0.47
OLL			57	0.57
LLL	55	0.32	57	0.69
PLeLe	57	0.42	—	—
OLeLe				
LLeLe	59	0.63	—	—
LeLeLe	61	0.78	—	—

* For abbreviations, see Table I.

where s is the number of saturated fatty acid acyls in a triglyceride molecule. The chromatographic separation was carried out as described for non-brominated triglycerides.

Table II (columns 4 and 5) shows that each polarity constant value K_3 corresponds to a different R_2' value, making possible the isolation and subsequent identification of all component triglycerides. As in the case of non-brominated glycerides, the partition coefficient of the bromides α_2' is a linear function of the polarity constant K_3 (Fig. 3).

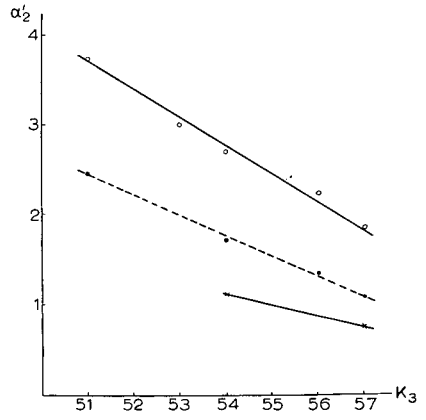


Fig. 3. Dependence of the partition coefficient of brominated triglycerides on the polarity constant K_3. O——O brominated triglycerides, $K_2 = 49$; ●----● $K_2 = 51$; ×——× $K_2 = 53$.

The chromatographic partition of brominated glycerides is of an additive nature. The following relationship exists between the partition coefficients of non-brominated and brominated triglycerides:

$$\alpha_2 = k \cdot \alpha_2' \cdot e \tag{7}$$

where $k = 0.75 \pm 0.10$ is a constant, determined by the nature of the halogen and the parameters of the reversed-phase system. The value of e is given by the expression

$$e = \alpha_2 / k \cdot \alpha_2' \tag{8}$$

Thus, for the identification of a triglyceride it is necessary to carry out its chromatographic separation before and after bromination. The polarity constant may be determined by reference to Fig. 2 and the number of double bonds is calculated from eqn. (8). Identification is accomplished by comparison of the data obtained with the theoretically possible composition of the triglycerides of the given polarity[11].

It is clear from the results that polar lipids (fatty acids) as well as non-polar lipids (triglycerides) follow MARTIN's rule for their partition in a reversed-phase system. There are, however, certain differences in the chromatographic behaviour of these lipids. The fact that the polarity constants of saturated and unsaturated fatty acids are equal does not necessarily imply equality of their partition coefficients, whereas the triglycerides strictly follow this rule. There is a linear relationship between polarity constant and logarithm of partition coefficient in the homologous series of fatty acids, but in the case of triglycerides it is the value of the partition coefficient itself that is a linear function of the polarity constant.

The experimental results seem to disagree with the data cited for other reversed-phase systems[7-9]. Further investigation is necessary to explain the reasons for this apparent difference.

SUMMARY

Reversed-phase chromatography studies have shown that the logarithm of the partition coefficient of saturated and unsaturated higher fatty acids and the partition coefficient of triglycerides are linear functions of the polarity constants of these lipids. By quantitative bromination of double bonds the polarity of triglycerides is increased. The reversed-phase chromatography of brominated glycerides makes possible the separation of otherwise inseparable triglyceride mixtures. The methods of chromatographic identification of lipids are discussed.

REFERENCES

1 A. J. P. MARTIN, *Biochem. Soc. Symposia, (Cambridge, Engl.)* 3 (1950) 4.
2 E. C. BATE-SMITH AND R. G. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.
3 H. VAN DUIN, *Biochim. Biophys. Acta*, 9 (1952) 580; 10 (1953) 198; 12 (1953) 489, 490.
4 G. J. PIEROTTI, C. H. DEAL, S. L. DELL AND P. E. PORTER, *J. Am. Chem. Soc.*, 78 (1956) 2989.
5 J. BOLDINGH, *Rec. Trav. Chim.*, 69 (1950) 247.
6 G. A. HOWARD AND A. J. P. MARTIN, *Biochem. J.*, 46 (1950) 532.
7 V. KOBRLE AND R. ZAHRADNIK, *Chem. Listy*, 48 (1954) 1189.
8 J. HIRSCH, *J. Lipid Res.*, 4 (1963) 1.
9 H. P. KAUFMANN *et al.*, *Fette, Seifen, Anstrichmittel*, 62 (1960) 153; 63 (1961) 125, 1021.
10 A. G. VERESHCHAGIN, *Biokhimiya*, 23 (1958) 721.
11 A. G. VERESHCHAGIN, *Biokhimiya*, 27 (1962) 866.
12 A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 35 (1941) 1358.
13 A. G. VERESHCHAGIN, S. V. SKVORTSOVA AND N. I. ISKHAKOV, *Biokhimiya*, 28 (1963) 868.

THE SIMULTANEOUS PAPER CHROMATOGRAPHIC SEPARATION OF PHOSPHATIDES, CEREBROSIDES AND SULFATIDES*

C. B. SCRIGNAR

Tulane University School of Medicine, Department of Medicine, New Orleans, La. (U.S.A.)

(Received July 30th, 1963)

INTRODUCTION

It has proved difficult to study the lipids in myelin because of the complexities involved in their separation. No paper chromatographic system has yet been described which separates phosphatides, cerebrosides and sulfatides simultaneously. This communication describes a new paper chromatographic system in which this is accomplished. This method utilizes silicic acid impregnated paper and a solvent system containing diisobutyl ketone, pyridine and water. Small quantities of lipid mixtures are separated accurately and rapidly.

MATERIALS AND METHODS

Brain (gray matter, white matter, and whole brain) from adult dog, fetal dog, rabbit, monkey, rat and beef and also peripheral nerves from frog, rat and rabbit were obtained immediately after death. The lipids were extracted by one or more of the following methods: (1) by the method of FOLCH *et al.*[1], (2) direct immersion of the tissue in chloroform–methanol, 2:1 v/v (20 vol. of solvent per g of tissue), (3) lyophilization of the tissue and extraction with chloroform–methanol, 2:1 v/v (20 vol. of solvent per g of tissue).

To aid in the interpretation of the chromatograms the following cerebral lipids were used as standards (those prepared in this laboratory were obtained from beef brain):

1. Phosphatidyl ethanolamine and phosphatidyl serine were isolated according to FOLCH's method[2].

2. Sulfatide and strandin samples were (a) obtained from J. FOLCH, and (b) prepared in our laboratory from beef brain as follows: strandin by the method of FOLCH, ARSOVE AND MEATH[3], and sulfatides by the method of LEES, FOLCH, SLOANE-STANLEY AND CARR[4].

3. Sphingomyelin preparation was obtained from E. KLENK AND M. HACK.

4. Cerebrosides were obtained from E. KLENK.

5. "Cephalin B" was prepared as described by BRANTE[5].

* Parts of this paper have been published in abstract form: C. B. SCRIGNAR AND V. J. FERRANS, The Paper Chromatography of Cerebrosides and Sulfatides of Brain Tissue, *Federation Proc.*, 19 (1960) 219. This study was supported by U.S.P.H.S. Grant, No. HTS 5133.

Impregnation of Whatman No. 1 and S & S 2043B filter paper with silicic acid and spot tests were done as described by HACK AND FERRANS[6].

The solvent system utilized consisted of diisobutyl ketone, pyridine and water in the ratio of 100:74:11. (The amount of water in the solvent system was critical and adjustments were necessary with each new batch of silicic acid impregnated paper; usually 9–13 parts of water were satisfactory for our purposes.) Two hundred ml of this mixture were sufficient for a 45 × 15 glass chromatographic cylinder. Five to twenty-five μl of lipid extract were applied to the chromatographic paper with a micropipette, making a spot about 1–2 mm in diameter. The spots were air-dried and the chromatograms developed for 4–12 h at 25°. The chromatograms were again air-dried, the solvent mixture washed out of the paper with two changes of 200 ml of 0.05 *M* H_2SO_3 and then stained for plasmalogens with fuchsin sulfurous acid, washed twice in 200 ml 0.05 *M* H_2SO_3 and counterstained with Rhodamin 6G. Duplicate runs were spot tested for free amino groups, choline and phosphorus.

Counterstaining with cresyl violet in 0.1% aqueous solution for one minute followed by extensive washing in 2% acetic acid was used frequently to demonstrate the metachromatic properties of myelin lipids.

RESULTS

Mobility of the lipids in the solvent system

The following lipids were identified in decreasing order of chromatographic mobility: cholesterol, cholesterol esters, and neutral lipids (as a single spot in the solvent front), cerebrosides, phosphatidyl serine, phosphatidyl ethanolamine, sulfatide, lecithins, sphingomyelin and a lipid close to the baseline which showed characterists of BRANTE's "cephalin B". Strandin was immobile in the system and stayed at the site of application. There was greater resolution of these lipids as the time of development increased.

TABLE I

RESULTS OF SPOT TESTS OF THE SEPARATED BRAIN LIPIDS

FSA—plasmalogen; $-NH_2$—ninhydrin; PHOS.—phosphorus; CHOLINE; META—presence of metachromasia when stained with cresyl violet; RHODAMIN—color of the lipid spots when viewed under U.V. light.

		FSA	*$-NH_2$*	*PHOS.*	*CHOLINE*	*META*	*RHODAMIN*
8	Cholesterol Neutral fat	o	o	o	o	o	yellow
7	Cerebrosides	o	o	o	o	o	light yellow
6	P. Serine	x	x	x	o	o	light orange
	P. Ethanolamine	x	x	x	o	o	light orange
5	Sulfatide	o	o	o	o	x	red-orange
4	Lecithin	x	o	x	x	o	bright yellow
3	Sphingomyelin	o	o	x	x	o	bright yellow
2	Unknown	o	o	x	o	x	red-orange
1	Strandin, etc.	o	o	o	o	x	red-orange

Identification of the lipids

The lipids were identified by comparing their mobilities with standard compounds simultaneously run on the same chromatograms. Individual spot tests for phosphorus, choline and free amino groups provided additional correlating information (see Fig. 1 and Table I).

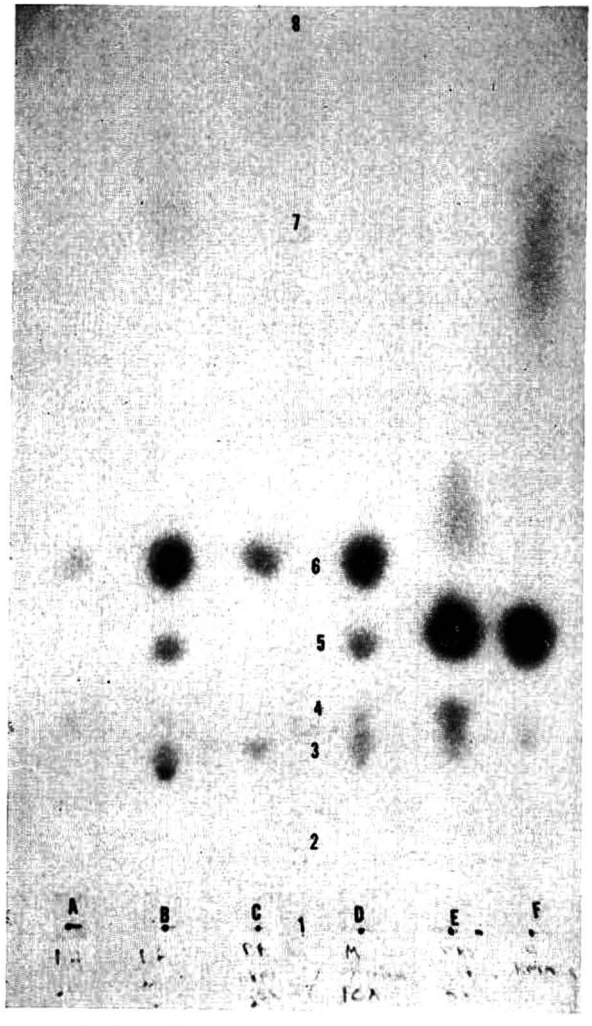


Fig. 1. Spot tests of brain lipids and standard compounds of A. Dog heart; B. Dog brain, white matter; C. Dog brain, gray matter; D. Monkey cerebellum; E. Crude sulfatide; F. Crude keracin.

Rhodamin 6G fluorescence

After staining with Rhodamin 6G, the developed chromatograms were washed several times with 0.05 *M* H_2SO_3 and viewed with an ultraviolet lamp, either wet or after drying. Washing is important to rid the paper of excess dye which sometimes alters the true fluorescing color of the stained lipid compounds. The cerebrosides, sphingo-

myelins and lecithins fluoresced a bright yellow. The sulfatide, strandin and "cephalin B" were a characteristic red-orange; the "cephalin B", however, was less intense due to its quantitative sparcity. When viewed with white light the sulfatides, strandin and "cephalin B" were distinguishable from the other lipids as their spots were red.

Staining with cresyl violet

Cresyl violet was used in place of Rhodamin 6G in many instances. The cerebrosides, sphingomyelins and lecithins stained light blue; phosphatidyl serine and ethanolamine were a darker shade of blue. Sulfatide, strandin, and "cephalin B" were a reddish-purple in sharp contrast to the rest of the lipids. After extensive washing with a 2 % solution of acetic acid, the silicic acid paper became almost colorless while the colors of the lipids remained stable. The metachromatic phenomenon will be amplified later in another communication.

DISCUSSION

The chromatographic system

There was no detectable hydrolysis of the various lipids even in the longer runs as compared with acetic acid systems where hydrolysis is a major hazard. All the lipids behaved in a stable manner and were easily identified even with 5–10 μl of a standard Folch extract, although 25 μl was perhaps optimal. Care was taken not to overload the paper chromatogram with the lipid extract as this produced streaking.

All chromatograms were developed at 25°. Chromatograms run at a lower temperature did not resolve the cerebrosides and sulfatides. These differences were probably caused by the solubility characteristics of these compounds at low temperatures.

Both S & S 204 3B and Whatman No. 1 filter paper were adequate for separation purposes, however, S & S 204 3B proved to be superior, showing minimal streaking with high concentrations of lipid extract, well delineated spots, good staining reaction and good silicic acid impregnation.

Cephalin fraction

Phosphatidyl ethanolamine and phosphatidyl serine had almost identical mobilities. However, when phosphatidyl serine was present in a sufficient quantity (accomplished by increasing the amount of lipid placed on the chromatogram or by placing some chemically isolated phosphatidyl serine on the same spot as that of normal brain tissue), the phosphatidyl serine had a faster mobility than the phosphatidyl ethanolamine.

"Cephalin B"

The identification of the lipid close to the baseline proved difficult. Its likeness to BRANTE's "cephalin B" was suggestive. This lipid exhibited metachromasia with cresyl violet, suggesting a chromogenic group on the molecule, perhaps similar to neurominic acid. Since this lipid also exhibited a positive phosphorus reaction, tentatively it appeared to have the characteristics attributed to "cephalin B".

APPLICATIONS

This chromatographic system has been a useful experimental tool for many problems. We have used this system to advantage in our laboratory in the following ways,

some of which will be elaborated on in a future communication: (1) determining which lipids are responsible for the metachromasia of myelin, (2) demonstrating the cerebrosides in the liver and spleen of a patient with Gaucher's disease, (3) determining the purity of chemically isolated lipids, (4) following the progression of lipids in column chromatography, and (5) providing semi-quantitative lipid information in degenerative and regenerative states of the nervous system.

SUMMARY

A paper chromatographic system utilizing silicic acid impregnated paper and a solvent system using diisobutyl ketone, pyridine and water has been described. Cerebrosides, phosphatidyl serine, phosphatidyl ethanolamine, sulfatide, lecithin, sphingomyelin, and an unknown lipid, possibly "cephalin B", were separated by this system. Strandin remained immobile while cholesterol, cholesterol esters and neutral lipids were located at the solvent front. The sensitivity, simplicity, and rapid lipid analysis are noteworthy. The versatility of the system is commented on and examples of its application are cited.

REFERENCES

1 J. FOLCH, M. LEES AND C. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 597.
2 J. FOLCH, *J. Biol. Chem.*, 146 (1942) 35.
3 J. FOLCH, S. ARSOVE AND J. B. MEATH, *J. Biol. Chem.*, 181 (1951) 819.
4 M. LEES, J. FOLCH, C. H. SLOANE-STANLEY AND S. CARR, *J. Neurochem.*, 4 (1959) 8.
5 G. BRANTE, *Acta Physiol. Scand.*, 15 (1949) suppl. 63.
6 M. H. HACK AND V. J. FERRANS, *Z. Physiol. Chem.*, 315 (1959) 157.

COMBINED COLUMN–PAPER CHROMATOGRAPHY

II. A NEW SYSTEM OF SOLVENTS FOR THE CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS

P. WIERZCHOWSKI AND D. KRUZE

Department of General Chemistry, Medical Academy, Warsaw (Poland)

(Received August 12th, 1963)

Difficulties in obtaining satisfactory separation of all amino acids of biological origin by means of paper chromatography have necessitated the application of two-dimensional systems. Nowadays this often consists of a combination of ionophoresis and chromatography. Recent adaptations of electrochromatography to new systems of solvents were described by BOTHMAN AND HIGA[1] and, as applied to the analysis of peptides, by RICHMOND AND HARTLEY[2].

The use of combined column–paper chromatography gave the desired separation and had, in addition, certain other advantages. Preliminary separation of amino acids on chromatographic columns and direct placing of the eluate from the columns on moving paper sheets are the principal characteristics of this method. The paper chromatograms are subsequently developed by the ascending method.

In our previous work[3], an apparatus and procedure were described for serial analyses of body fluids. In the present work, we describe further modifications of the procedure and the results of analysing amino acids present in blood, urine and cerebrospinal fluid.

EXPERIMENTAL

Apparatus

(1) Chromatographic columns 0.9 by 45 cm filled with starch.

(2) Containers 100 ml in volume connected with particular columns directly or by means of igelite tubes.

(3) An automatic device for moving sheets of paper at a rate of 0.75 cm/h (*i.e.* 54 cm, the length of a paper sheet, during a 72-hour period). Columns, from which the eluate flows on to single paper sheets that are moving along, are fastened to this device. To evaporate an eluate deposited on the paper, a stream of warm air is blown under each column.

(4) Bell glasses for ascending chromatography.

Reagents, solvents and adsorbents

(1) Ninhydrin reagent consisting of 0.5 g of ninhydrin dissolved in 100 ml of absolute ethanol with the addition of 0.5 ml of 1 *N* NaOH.

(2) Alcohol solutions: (a) 10 % (v/v) aqueous solution of ethanol, (b) 10 % acidic solution of ethanol (up to 90 ml of distilled water, 10 ml of ethanol and 1.0 ml of 1 *N* HCl), (c) 90 % (v/v) aqueous solution of ethanol, (d) 98 % (v/v) aqueous solution of methanol.

(3) 10 % (v/v) aqueous solution of ammonia.

(4) Potato starch, thoroughly washed with water, dried in air, ground in a mortar and sieved.

(5) Whatman No. 4 paper.

(6) Amberlite IR-120, Wofatite KPS-200, or other sulphonated cation exchangers.

Procedure

Preparing columns

Ethanol (90 %) is added to starch in such a quantity as to form a thick suspension after thorough mixing. This suspension is subsequently poured into the columns up to the point where the level of starch reaches the desired height (40 cm). A column filled with starch is washed with an acidified 10 % solution of ethyl alcohol until the eluate flowing out of it begins to show an acidic reaction.

The adsorbent in a column can be acidified by a previously prepared acidic solution of alcohol. However, in order to save time, it is better to apply a more strongly acid solution (*e.g.*, 0.5 ml of concentrated HCl in 10 ml of 10 % (v/v) ethyl alcohol). The excess of acid is subsequently washed out of the column by passing 20 ml of 10 % ethyl alcohol through it.

The solution should flow out of the column at a rate of 14–18 ml per day. This can be achieved by appropriate tamping of the starch in the column, as well as by placing the solvent container at various heights above the column. When analysing samples containing a large number of amino acids, or biological material in which the presence of many other ninhydrin-positive compounds may be expected, the rate of elution should be adjusted to about 14 ml per day.

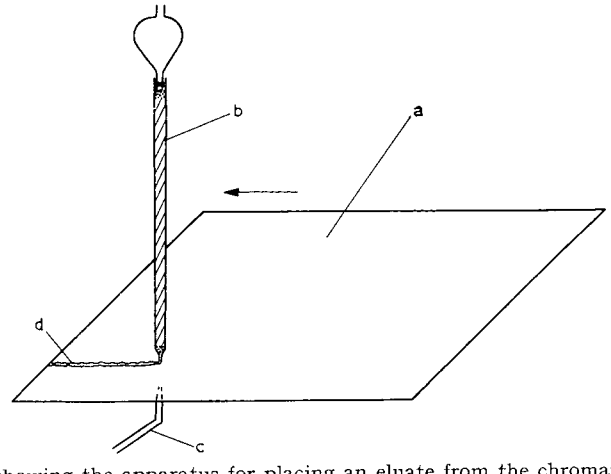


Fig. 1. Diagram showing the apparatus for placing an eluate from the chromatographic column on a moving paper sheet. (a) sheet of paper; (b) column; (c) tube blowing warm air under the column outlet; (d) trace formed by the eluate which dropped and dried.

Separation

From 0.3 to 3.0 mg of a mixture of amino acids dissolved in a small volume (max. 1.5 ml) of 10 % ethyl alcohol is placed in the column prepared in the manner described above. Immediately after the solution has soaked in, about 0.5 ml of 10 % ethyl alcohol is added to rinse the internal walls of the column and, when this portion has flowed down to the adsorbent, the column should be connected to a vessel containing 100 ml of 10 % ethanol. In the final stage of the outflow of this solvent (on the sixth day), 110 ml of 10 % ethanol with 1 ml of 1 *N* hydrochloric acid solution are added to the container. From the moment the amino acid mixture is added until the outflow of the acidified 10 % ethanol, the lower end of the column should touch a sheet of paper moving beneath it (Fig. 1).

The separation on the column lasts 13 days and the eluate is collected on 4 sheets of paper. After the column separation is finished, these sheets, on which the fractions have been automatically placed in the form of a narrow band, are subjected to ascending chromatography in 98 % methanol.

Preparation of biological material

Prior to chromatographic analysis, protein, inorganic salts and non-polar compounds which interfere with the chromatographic separation of amino acids, should be removed from the biological material. To this end, ethanol was added to serum, urine or other biological fluid up to a final concentration of 60 % (v/v). The protein precipitated was filtered off and washed twice with small portions of 60 % ethanol. The combined filtrates were diluted with an equal volume of water and desalted by passing through on Amberlite IR-120 ion exchanger, using the generally accepted technique. Amino acids bound on the ion exchanger were eluted with 60 ml of 6 *N* ammonia, the eluate thus formed was evaporated to dryness on a water bath, *ca.* 0.5 ml of distilled water was added and the mixture was evaporated once more. The dry residue was dissolved in 1 ml of 10 % ethanol and transferred to a starch column.

RESULTS

Standard mixture

In using column–paper chromatography, about 1.5 mg of a standard mixture of amino acids, dissolved in 1 ml of 10 % ethanol, were subjected to separation. The quantities of the particular amino acids varied in this mixture and fluctuated between 0.0025 and 0.017 mmoles. After staining with ninhydrin, chromatograms were obtained on which the amino acids formed distinct, clearly separated spots, except α-aminobutyric acid, which partially overlapped methionine, and ornithine, which touched lysine (Fig. 2). The distribution of all spots was characteristic and occurred repeatedly in several experiments. Slight irregularity was shown only by glutamic and aspartic acids, which tended to elute down the column slightly faster than other compounds. These minor differences did not, however, cause overlapping of spots and did not present difficulties in identification.

Serum

Nine millilitres of human serum were analysed in the protein-free and desalted form. The presence of many compounds stained with ninhydrin was observed on the chro-

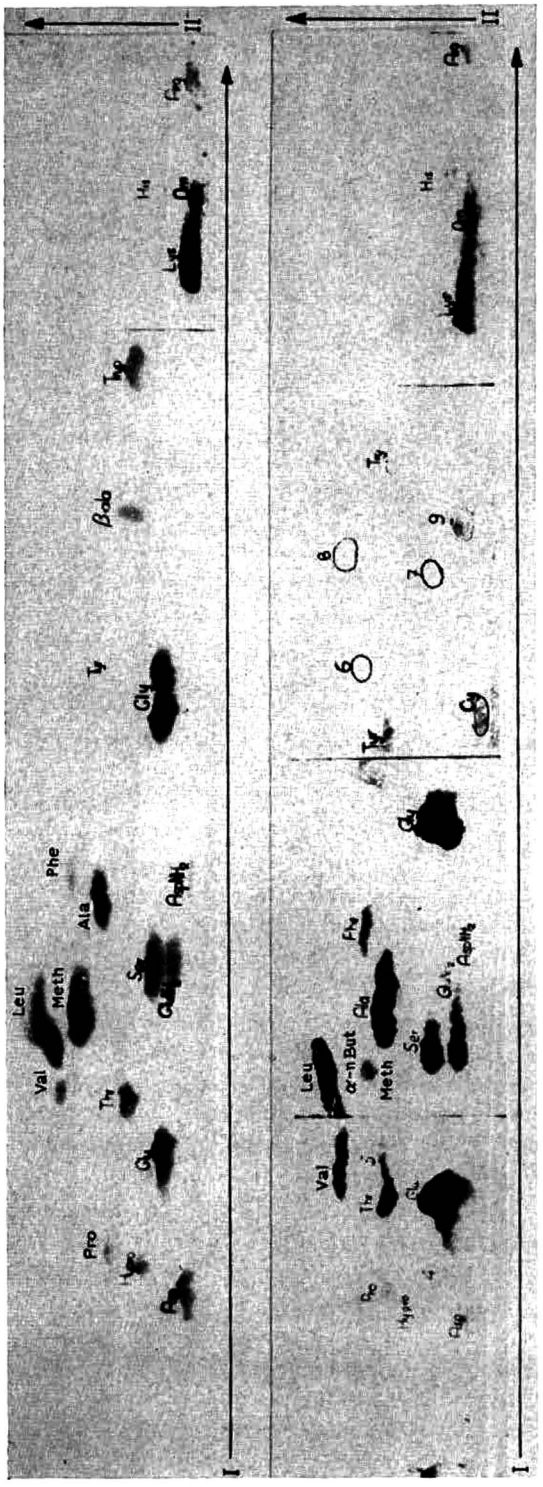


Fig. 2. Chromatogram of a standard mixture of amino acids. (I) direction of placing the eluate from the column; (II) ascending chromatography. Abbreviations: Asp = aspartic acid; Hypro = hydroxyproline; Pro = proline; Glu = glutamic acid; Thr = threonine; Val = valine; Leu = leucine; Meth = methionine; α-n But = α-aminobutyric acid; Ser = serine; Glu NH_2 = glutamine; Ala = alanine; Phe = phenylalanine; Asp NH_2 = asparagine; Gly = glycine; Tyr = tyrosine; β-ala = β-alanine; Tryp = tryptophan; Lys = lysine; Orn = ornithine; His = histidine; Arg = arginine.

Fig. 3. Chromatogram of amino acids contained in human serum. Abbreviations as in Fig. 2. 1,2,3,4,5,6,7,8,9 unidentified spots.

matograms (Fig. 3). Amongst them, in addition to the amino acids occurring in large quantities in serum (glycine, glutamic acid, glutamine), there were also amino acids that are present in low concentration (hydroxyproline, aspartic acid, asparagine, α-amino-*n*-butyric acid). Besides amino acids, spots were recorded corresponding to other chemical compounds (in the photographs, they are denoted by the numbers 1–9).

Urine

The chromatogram shown in Fig. 4 was obtained from 3 ml of the urine of a healthy adult individual. An almost full spectrum of amino acids was recorded here despite the fact that they occurred in considerably varying concentrations. In addition, the presence of 17 other ninhydrin-positive compounds, most of them located in places distant from the spots formed by amino acids, were visible on the chromatogram.

Cerebrospinal fluid

Since the concentration of free amino acids is lower in cerebrospinal fluid than in blood and urine, 45 ml of a fluid obtained from a lumbar puncture of an individual suffering from epilepsy was subjected to a single analysis. Clear and distinctly separated spots, of which 19 corresponded to common amino acids and 8 were formed by unidentified compounds, were recorded on the chromatogram (Fig. 5).

DISCUSSION

The two-dimensional chromatograms obtained by combined column–paper chromatography are similar as those in the coloured photographs shown by FOWDEN[4]. The difference between his method and ours is that, instead of the toilsome work of transferring substances collected in numerous fractions on a collector, to the paper, we used an apparatus[3] which directly places the eluate of several analyses on the sheets simultaneously. This method extends the possibilities of using various adsorbents and various solvent systems for developing chromatograms.

Chromatograms of the analysis of one mixture only are placed on 2–4 sheets of paper. Under such conditions, particular components are separated into clearly defined spots by the use of a larger quantity of a mixture of amino acids (0.3–3 mg) than in two-dimensional paper chromatography. This enables us to detect components occurring in a quantity of a few μg, as well as those occurring in excess (> 300 μg). The results depend on the selection of suitable solvents. In the present work, two solvents, 10 % ethanol and 10 % ethanol with 1 ml of 1 *N* hydrochloric acid, were used for separating amino acids on the column; the eluate was collected on 4 sheets of paper. The chromatograms were subsequently developed with methanol by the ascending method. Quantitative values of particular components were determined by the method of elution and colorimetry developed by KAY *et al.*[5].

This method is easier in practice compared with the procedure previously described[3], since it does not require a concentration gradient of solvents and gives a more accurate separation of neutral amino acids. In particular, glycine separates from serine and phenylalanine from methionine, while proline and hydroxyproline appear at some distance from the remaining neutral amino acids. This is important, as there is only slight staining of imino acids with ninhydrin.

With the intention of separating as many components as possible during a

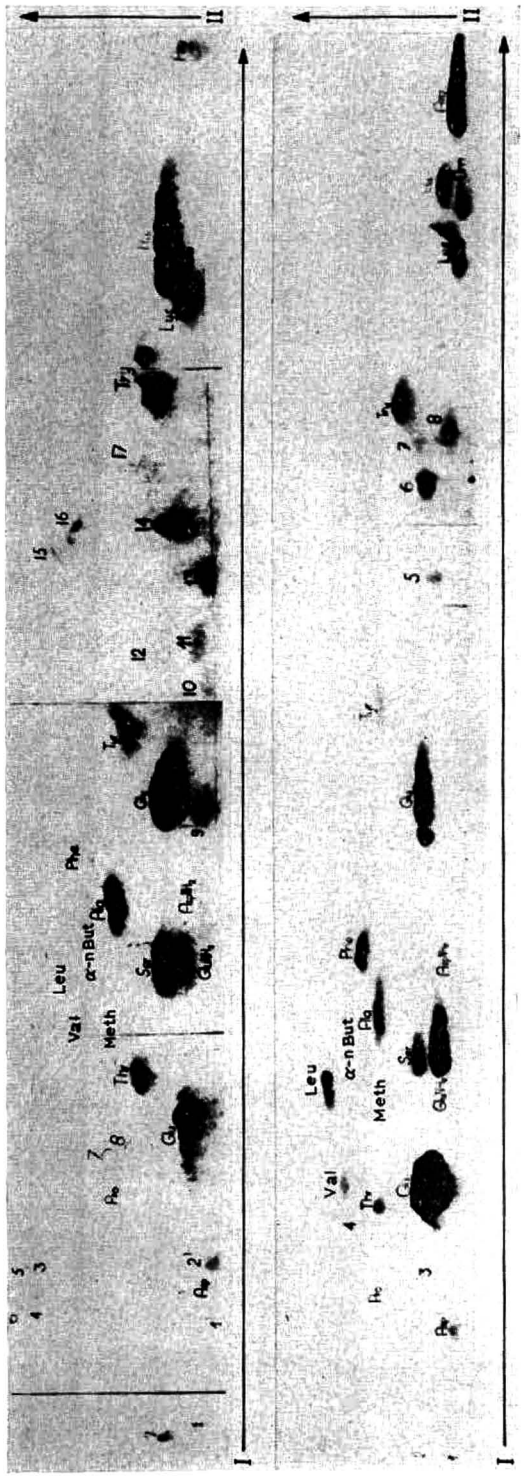


Fig. 4. Chromatogram of amino acids contained in human urine. Abbreviations as in Fig. 2. 10,11,12,13,14,15,16,17 unidentified spots.
Fig. 5. Chromatogram of amino acids contained in human cerebrospinal fluid. Abbreviations as in Fig. 2.

single analysis, we conducted the development on a column over a 13-day period, collecting the eluate on 4 sheets of paper. This long developing period is, however, used simultaneously for making larger numbers of serial analyses. By means of an appropriate device, an eluate from 10 or even more columns is simultaneously placed on particular paper sheets.

In addition to amino acids, the spots of new compounds denoted by Fig. 3–5 were discovered on chromatograms of urine and other biological fluids, this enabled us to begin their identification. Preliminary investigations, would indicate that some of these spots come from amino alcohols, amino sugars and peptides.

SUMMARY

The systems of solvents and the procedure have been elaborated in order to obtain two-dimensional chromatograms of amino acids by the combined column–paper chromatography method. The eluate from the columns was directly placed on sheets of paper by means of appropriate apparatus. Chromatograms were obtained for a quantitative determination of amino acids contained in blood, urine and cerebrospinal fluid. In addition to amino acids, clearly separated spots of other amino derivatives, such as peptides and amino alcohols, were also obtained on chromatograms.

REFERENCES

1 F. BOTHMAN AND A. HIGA, *Anal. Biochem.*, 3 (3) (1962) 173.
2 V. RICHMOND AND B. S. HARTLEY, *Nature*, 184 (1959) 1869.
3 P. WIERZCHOWSKI, I. JANCZARSKI AND D. KRUZE, *Acta Biochim. Polon.*, 9 (1962) 343.
4 L. FOWDEN, *Endeavour*, 21 (1962) 40.
5 R. E. KAY, D. C. HARRIS AND C. ENTENMAN, *Arch. Biochem. Biophys.*, 63 (1956) 14.

CHROMATOGRAPHIC SEPARATION OF PYRIDOXYL DERIVATIVES OF AMINO ACIDS*

CARLO TURANO, PAOLO FASELLA, PAOLA VECCHINI AND ANNA GIARTOSIO
Istitutò di Chimica Biologica, Università di Roma e Centro di Enzimologia del C.N.R., Rome (Italy)

(Received August 5th, 1963)

Much interest has recently been given to pyridoxyl derivatives of amino acids, because it is in this form that coenzyme-binding sites and coenzyme-substrate complexes have been isolated from pyridoxal dependent enzymes.

Even though methods for the synthesis of pyridoxyl-amino acids are known[1], no procedure is yet available for the quick identification of less then micromolar amounts of these compounds. It is the purpose of this paper to describe a two-dimensional chromatographic technique that fulfils this requirement.

The non-phosphorylated form of the pyridoxyl derivatives was chosen for the present work because it is this form which is obtained after hydrolysis of reduced pyridoxal enzymes.

EXPERIMENTAL

Preparation of α-pyridoxyl-amino acids

Twenty milligrams of the amino acid were dissolved in 1.5 ml of hot water. Solutions of dicarboxylic acids were neutralized by the addition of solid $KHCO_3$. The aqueous solution of the amino acid was diluted with an equal volume of methanol, 2 mg of solid pyridoxal hydrochloride were added and the solution was neutralized. The solution acquired a characteristic yellow colour indicating Schiff base formation between the aldehydic group of pyridoxal and the α-amino group of the amino acid[2]. The yellow solution was mixed with 5–10 mg of sodium borohydride. This treatment produced a reduction of the azomethinic double bond and was accompanied by the disappearance of the yellow colour.

The solution was deposited as a 10 cm long band, 7 cm from the end of a sheet of Whatman 3 MM paper, and developed for 20 h by descending chromatography in *n*-butanol–acetic acid–water (40:10:50).

Examination of dried chromatograms in U.V. light revealed the presence of three bright-blue fluorescent spots of decreasing intensity corresponding respectively to the pyridoxyl-amino acid compound, to pyridoxine and to pyridoxamine. A small vertical strip of the chromatogram was cut off and treated with ninhydrin to locate the amino acids.

The band corresponding to the pyridoxyl-amino acid was cut from the chromatogram, eluted with water and the eluate was submitted to two-dimensional paper

* This work is part of a programme supported by "Impresa di Enzimologia del Consiglio Nazionale delle Ricerche"

chromatography using collidine–lutidine (1:1) saturated with water for the first and *n*-butanol–acetic acid–water (40:10:50) for the second run.

All pyridoxyl-amino acid compounds tested in this way gave a single spot.

The chromatograms were analyzed in U.V. light and sprayed with ninhydrin to detect free amino groups or with 2,4-dichloroquinone-chlorimide, to detect the phenolic groups of the pyridoxyl derivatives[3].

Preparation of pyridoxyl-lysine derivatives

α,ε-Dipyridoxyl-lysine was prepared (together with α-pyridoxyl-lysine) as follows: 8 mg of lysine dihydrochloride and 24 mg of pyridoxal hydrochloride were dissolved in 0.5 ml of water and neutralized with dilute NaOH. The solution was then evaporated over a gentle heat and to the residue, dissolved in methanol, was added sufficient sodium borohydride to completely discharge the yellow colour. After filtration the solution was applied as a long band on a sheet of Whatman 3 MM paper and analyzed in the usual way.

ε-Pyridoxyl-lysine was prepared as follows: 300 mg of lysine dihydrochloride were dissolved in 2 ml of water and an excess of solid basic copper carbonate was added. The insoluble matter was filtered off on sintered glass and the filtrate was gently heated to dryness in an evaporating basin.

The dry residue was suspended in 5 ml of methanol containing 50 mg of pyridoxal. The suspension was heated at 40° for a few minutes, cooled to room temperature, treated with several aliquots of sodium borohydride and the black copper precipitate was filtered off. The solution was concentrated under vacuum, submitted to preparative chromatography and analyzed as described for the other pyridoxyl-amino acids. The spot corresponding to ε-pyridoxyl-lysine was identified by its fluorescence and by the colour reaction described by OLESEN LARSEN AND KJAER[4] for differentiating α- from ω-amino acids.

α-Pyridoxyl-lysine was prepared as follows: 50 mg of pyridoxal hydrochloride and 100 mg of ε-acetyl-lysine[5] were dissolved in 5 ml 50% ethanol and neutralized with dilute NaOH. The mixture was then dried under vacuum. A suspension of 20 mg sodium borohydride in 2 ml of ethanol was added to the dry residue and, after 10 min, 1 ml of 1 *M* acetic acid. The mixture was dried under vacuum, dissolved in 2 ml of 6 *N* HCl and sealed in a vial. The vial was kept at 120° for 7 h. The excess of acid was then removed under vacuum. The dry residue was dissolved in a minimum of water and analyzed as usual.

RESULTS

The R_F values in different solvents of the pyridoxyl amino acids investigated in the present paper are reproduced in Table I.

A typical two-dimensional chromatogram of a mixture of the derivatives under investigation is reproduced in Fig. 1, where the fluorescent spots which appear upon exposure to U.V. light are shown.

As regards the identification of the lysine derivatives, ε-pyridoxyl-lysine gave the expected positive ninhydrin reaction but showed no ninhydrin reaction after treatment with the OLESEN LARSEN AND KJAER reagent[4], which blocks α-amino groups.

When lysine was reacted with an excess of pyridoxal (see preparation of α,ε-dipyridoxyl-lysine), two pyridoxyl derivatives were obtained, one of which, with R_F

TABLE I

R_F VALUES OF PYRIDOXYL-AMINO ACIDS IN VARIOUS SOLVENTS

Pyridoxyl-amino acid	R_F *values*	
	in collidine–lutidine (1 : 1) saturated with water	*in n-butanol–acetic acid–water (40 : 10 : 50)*
Alanine	0.56	0.39
Arginine	0.28	0.06
Aspartic acid	0.41	0.19
Phenylalanine	0.57	0.62
Glycine	0.54	0.22
Glutamic acid	0.30	0.32
Histidine	0.54	0.07
Leucine	0.72	0.52
Lysine (ε-pyridoxyl)	0.45	0.06
Lysine (α-pyridoxyl)	0.24	0.13
Lysine (α,ε-dipyridoxyl)	0.47	0.09
Methionine	0.70	0.40
Serine	0.59	0.20
Threonine	0.77	0.31
Tyrosine	0.83	0.38
Valine	0.74	0.49
Pyridoxine	0.88	0.63
Pyridoxamine	0.52	0.45

All values were obtained on Whatman No. 4 paper by descending chromatography.

values of 0.47 and 0.09 in collidine–lutidine and butanol–acetic acid respectively, was ninhydrin negative and was identified as α,ε-dipyridoxyl-lysine. The other derivative, with R_F values of 0.25 and 0.13 respectively, was ninhydrin positive, even after treatment with the OLESEN LARSEN AND KJAER reagent[4], and was identified as α-pyridoxyl-lysine; the same spot was obtained starting the preparation with ε-acetyl-lysine (see EXPERIMENTAL).

DISCUSSION

The chromatographic procedure described in this paper provides a simple and quick way of identifying 0.01 micromolar amounts of 16 pyridoxyl-amino acids.

The method could therefore prove useful in the investigation of the apoenzyme-coenzyme and enzyme-substrate complexes of pyridoxyl phosphate dependent enzymes. Application of this method to glutamic-aspartic transaminase from pig heart will be described elsewhere.

It is interesting to observe that owing to the presence of the pyridoxyl group in the molecules of the amino acid derivatives, the R_F values are, as was to be expected, slightly higher then those of the corresponding amino acids in the same solvents.

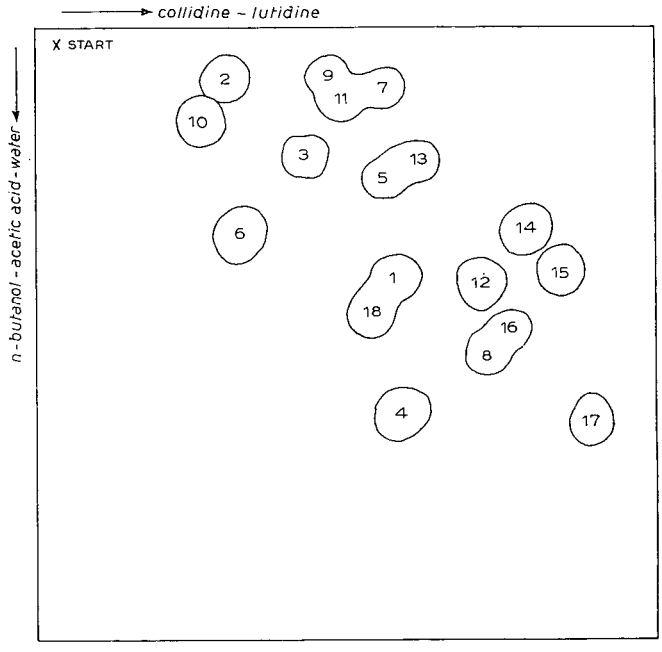


Fig. 1. The fluorescent spots appearing on a typical chromatogram of a mixture of the pyridoxyl-derivatives of the following amino acids: 1 = alanine; 2 = arginine; 3 = aspartic acid; 4 = phenylalanine; 5 = glycine; 6 = glutamic acid; 7 = histidine; 8 = leucine; 9 = lysine (ε-pyridoxyl); 10 = lysine (α-pyridoxyl); 11 = lysine (α,ε-dipyridoxyl); 12 = methionine; 13 = serine; 14 = threonine; 15 = tyrosine; 16 = valine; 17 = pyridoxine; 18 = pyridoxamine.

SUMMARY

The preparation of sixteen pyridoxyl amino acid compounds is described, together with their separation by means of a two-dimensional chromatographic system.

REFERENCES

[1] D. HEYL, S. A. HARRIS AND K. FOLDERS, *J. Am. Chem. Soc.*, 70 (1948) 3429;
W. B. DEMPSEY AND H. N. CHRISTENSEN, *J. Biol. Chem.*, 237 (1962) 1113;
E. H. FISCHER, *et al.*, in E. E. SNELL, *et al.* (Editors), *I.U.B. Symposium on Pyridoxal Catalysis*, Pergamon, Oxford, 1963, p. 543.
[1] P. FASELLA, C. TURANO AND A. GIARTOSIO, unpublished data.
[2] D. E. METZLER AND E. E. SNELL, *J. Am. Chem. Soc.*, 74 (1952) 979.
[3] F. FEIGL AND E. JUNGREIS, *Clin. Chim. Acta*, 3 (1958) 399.
[4] P. OLESEN LARSEN AND A. KJAER, *Biochim. Biophys. Acta*, 38 (1960) 148.
[5] A. NEUBERGER AND F. SANGER, *Biochem. J.*, 37 (1943) 515.

PAPER CHROMATOGRAPHIC DETECTION AND ESTIMATION OF L-AMINO ACID OXIDASE OF SNAKE VENOM

R. W. P. MASTER*

The Haffkine Institute, Parel, Bombay (India)

(Received August 13th, 1963)

The L-amino acid oxidase activity in snake venoms is usually estimated by any of the following methods: (a) manometric method where oxygen consumption is measured, (b) estimation of ammonia produced, (c) colorimetric estimation of keto acid produced by the L-amino acid oxidase in presence of catalase and (d) the estimation of hydrogen peroxide produced in absence of catalase[1]. Recently, WEISSBACH and coworkers[2] have described a spectrophotometric procedure for the determination of L-amino acid oxidase in snake venoms. BOMAN AND KALETTA[3] have used a filter paper spot test, where remaining L-leucine is determined after oxidation with the snake venom enzyme, by the ninhydrin reaction.

While working on the enzyme systems in the venoms of Indian snakes, a paper chromatographic method was developed to detect and estimate L-amino acid oxidase. This method can also be used for the detection and determination of D-amino acid oxidase and histidase.

In the absence of catalase, L-amino acid oxidase acts on L-amino acids according to the following scheme[1].

$$RCH(NH_2)COOH + O_2 + H_2O \rightarrow RCOCOOH + NH_3 + H_2O_2$$

$$RCOCOOH + H_2O_2 \rightarrow RCOOH + CO_2 + H_2O.$$

With L-histidine as the substrate, the product of reaction would be imidazole acetic acid according to the reaction given above. The imidazole acetic acid formed is detected and estimated over the range 5–40 μg using diazotized sulphanilic acid as the spray reagent.

PROCEDURE AND RESULTS

Materials and methods

Venoms. The lyophilized venoms of cobra and Russell's viper collected at the Haffkine Institute were used. L-Histidine hydrochloride was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, urocanic acid was kindly supplied by Dr. MARIAN W. KIES of N.I.H. (U.S.A.) and imidazole acetic acid was a gift from Dr. HERBERT TABOR, (N.I.H.) U.S.A. A 1% solution of the lyophilized venom in distilled water was prepared. Aqueous solutions (2 mg/ml) of L-histidine hydrochloride,

* Present address: Division of Enzymology, Institute of Medical Research, The Chicago Medical School.

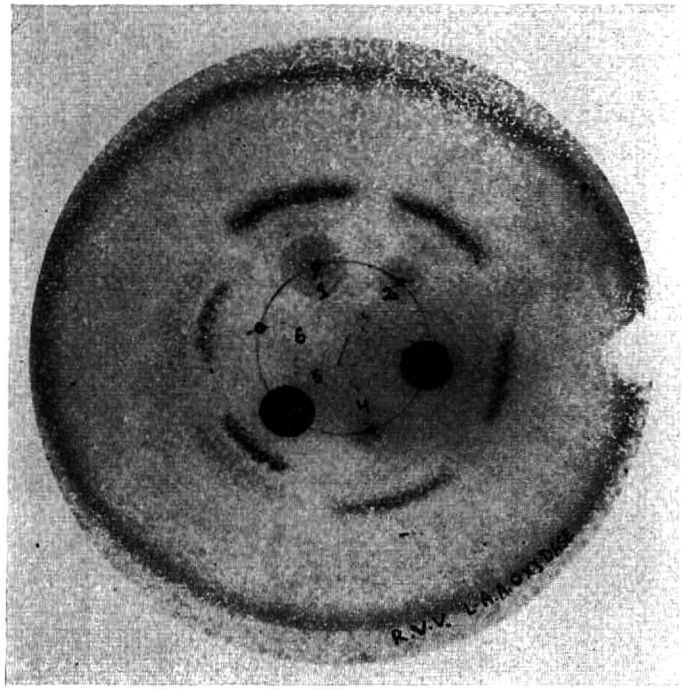


Fig. 1. The circular paper chromatogram showing the detection of L-amino acid oxidase in the venom of Russell's viper. Solvent = *n*-Butanol–acetic acid–water (4:1:5).

imidazole acetic acid and urocanic acid were used for spotting different volumes of those compounds on paper chromatograms. Circular paper chromatography was carried out according to the method of GIRI AND RAO[4].

Usually the reaction was carried out with 1 ml (2 mg/ml) L-histidine hydrochloride solution, 1.0 ml of 0.05 *M* Tris–HCl buffer pH 8.5 and 1.0 ml of 1 % solution of the venom under investigation. After incubation at 37° for 12–14 h, 10 μl aliquots of the reaction mixture were used for the chromatographic procedure. Two solvents were found to be useful for paper chromatography: solvent I = 77 % ethyl alcohol, solvent II = *n*-butyl alcohol–acetic acid–water, (4:1:5, v/v/v).

The spray reagent used was diazotized sulphanilic acid (Pauli's Reagent) prepared according to the method of MANN AND LEONE[5].

TABLE I

R_F VALUES OF L-HISTIDINE HYDROCHLORIDE, IMIDAZOLE ACETIC ACID AND UROCANIC ACID OBTAINED UNDER THE EXPERIMENTAL CONDITIONS DESCRIBED IN THE TEXT

	L-Histidine HCl	*Imidazole acetic acid*	*Urocanic acid*
Solvent I (77 % ethyl alcohol)	0.56	0.67	0.71
Solvent II (*n*-butyl alcohol–acetic acid–water) (4:1:5)	0.31	0.54	0.71

A typical chromatogram obtained is illustrated in Fig. 1.

The R_F values obtained for histidine, imidazole acetic acid and urocanic acid on a circular paper chromatogram using a 24 cm diameter disc of Whatman No. 1 filter paper are given in Table I.

With the solvents used, and especially with solvent II, imidazole acetic acid and urocanic acid can be distinctly separated from L-histidine hydrochloride. Thus even histidase which gives urocanic acid[6] with L-histidine as the substrate can be detected by this method in presence of the L-amino acid oxidase. As the D- and L-isomers of histidine have the same R_F values, even D-amino acid oxidase could be detected by the same procedure.

The colour of the bands for all three compounds is reddish brown on paper chromatograms. It is remarkably stable and the chromatograms can be preserved in the dark for months without any apparent fading of the bands. Of the several solvents tried to elute the coloured products from paper chromatograms, glass distilled water was found to be most satisfactory. 3 ml of glass distilled water could extract all the colour from the bands in 20–30 min. After elution of the colour from filter paper, the solution was yellowish red and could be measured colorimetrically.

Coloured complexes of all the three compounds had a maximum absorption at 360 mμ in the visible region of the spectrum as determined in a Unicam spectrophotometer Sp. 600. The original imidazole compounds had no absorption at this wave length. Hence the absorption of coloured products of both imidazole acetic acid and urocanic acid with Pauli's reagent can be measured and can be utilized for quantitative determination of L-amino acid oxidase as well as histidase. Imidazole acetic acid could be measured over a range of 5 to 40 μg and urocanic acid in the range of 5 to 60 μg under the conditions of the experiment described above.

As soon as the reaction mixture is applied to the paper chromatogram and dried, further enzymic reaction stops and hence the extent of oxidation of the substrate can be followed both qualitatively as well as quantitatively.

This method was routinely employed in our laboratory to detect and estimate L-amino acid oxidase in Indian snake venoms and also that in the white and yellow venoms of *Vipera ammodytes* after electrophoretic and chromatographic fractionation.

Another way of comparison of activities in venoms on a semiquantitative basis has been to compare the concentrations of venoms to produce a minimum visible reaction on paper chromatograms or the minimum concentrations of different venoms required to convert all the substrate into imidazole acetic acid.

DISCUSSION

As observed by paper chromatography, since L-amino acid oxidase converts all of L-histidine hydrochloride into imidazole acetic acid, the reaction can be used for the biological preparation of imidazole acetic acid following the procedure of MEISTER[7].

Histamine hydrochloride and L-histidine hydrochloride have the same R_F values in the *n*-butyl alcohol–acetic acid–water solvent. Hence if L-histidine decarboxylase is present in the reaction mixture, its presence will not interfere with L-amino acid oxidase activity determinations.

Though L-histidine is relatively less susceptible to the action of L-amino acid oxidase of snake venoms as compared to L-leucine[1], the method described in this

communication has the added advantage in that the product of reaction distinctly gets separated from the substrate on paper chromatograms, thus affording a quick identification of that enzyme qualitatively as compared to the method of BOMAN AND KALETTA[3], where the disappearance of the ninhydrin reacting spot is the criterion of the activity.

ACKNOWLEDGEMENTS

The author is grateful to Dr. S. S. RAO and Dr. H. I. JHALA, for their encouragement and to the Government of India for the award of a fellowship.

The work was carried out in the Immunology Department, Haffkine Institute, Bombay.

SUMMARY

A paper chromatographic method is described for the detection and estimation of L-amino acid oxidase in snake venoms using L-histidine as the substrate. The product of reaction is separated paper chromatographically using 77 % ethyl alcohol or *n*-butyl alcohol–acetic acid–water. The method can also be applied to the detection and determination of histidase and D-amino acid oxidase. Histidase and L-histidine decarboxylase do not interfere in the determination of L-amino acid oxidase.

REFERENCES

1 E. A. ZELLER, *Advan. Enzymol.*, 8 (1948) 477.
2 H. WEISSBACH, A. V. ROBERTSON, B. WITKOP AND S. UDENFRIEND, *Anal. Biochem.*, 1 (1960) 286.
3 H. G. BOMAN AND U. R. KALETTA, *Biochim. Biophys. Acta*, 24 (1957) 619.
4 K. V. GIRI AND N. A. N. RAO, *Nature*, 169 (1952) 923.
5 T. MANN AND E. LEONE, *Biochem. J.*, 53 (1953) 140.
6 H. TABOR AND A. H. MEHLER, *Methods Enzymol.*, 2 (1955) 228.
7 A. MEISTER, *Venoms*, Publication No. 44 of American Association for Advancement of Science, Washington, D.C., 1956, p. 295.

CONTRIBUTION A L'ANALYSE DES POLYÉTHYLÈNEGLYCOLS ET DE LEURS MONOÉTHERS PAR CHROMATOGRAPHIE DE PARTAGE DE LEURS ESTERS 3,5-DINITROBENZOÏQUES

I. POLYÉTHYLÈNEGLYCOLS

H. GAUTHIER

Laboratoire Analytique, Laboratoire Central, FMC-SA., Saint-Denis, Seine (France)*

ET

G. MANGENEY

*Chef Service PAT**, Laboratoire Central, FMC-SA.*, Saint-Denis, Seine (France)*

(Reçu le 3 juillet 1963)

La méthode de chromatographie sur papier des 3,5-dinitrobenzoates (DNB) des polyéthylèneglycols (PEG) et de leurs éthers avec les alcools aliphatiques, publiée par BORECKÝ ET GASPARIČ[1,2], a été appliquée dans notre laboratoire à l'étude des produits techniques obtenus par polyoxyéthylénation du glycol ("polyéthylèneglycols"). Nous rappelons que, selon les auteurs cités, les substances (PEG, respectivement leurs dérivés) sont transformées préalablement en 3,5-dinitrobenzoates, lesquels sont chromatographiés sur papier imprégné, selon les cas, de formamide, diméthylformamide, huile de paraffine. La révélation est obtenue par transformation, sur le papier, en bases de Schiff correspondantes (de couleur jaune), grâce à une réduction au moyen de chlorure stanneux suivie de condensation avec le *p*-diméthylaminobenzaldéhyde.

La méthode nous a effectivement fourni de bons résultats. Cependant, dans le cas des diesters 3,5-dinitrobenzoïques des polyéthylèneglycols, et avec le formamide comme phase stationnaire, nous avons rencontré des difficultés de séparation (forme allongée des taches, défaut de reproductibilité, etc.). Il est apparu, de plus, au cours du travail, que la phase stationnaire (formamide) gênait la révélation.

Nous avons également étudié l'application du principe de la méthode de BORECKÝ ET GASPARIČ, à savoir la chromatographie sur papier à l'état d'esters 3,5-dinitrobenzoïques, à la classe fort importante mais, à notre connaissance, non traiteé par les auteurs tchèques, des alkylphénols polyoxyéthylénés. Mentionnons seulement pour l'instant que l'emploi des systèmes de phases préconisés par BORECKÝ ET GASPARIČ, tant pour le cas des DNB des polyéthylèneglycols que pour celui des DNB des alcools aliphatiques polyoxyéthylénés (notamment l'utilisation d'huile de paraffine comme phase stationnaire), n'a pas donné de résultat positif. Par contre, la résolution des DNB des alkylphénols polyoxyéthylénés (du moins de leurs douze à quinze premiers

* Française des Matières Colorantes, Société Anonyme.
** Produits Auxiliaires de Teinture.

termes) a pu être réalisée grâce à l'emploi d'une phase stationnaire telle que l'huile de soja, ayant un caractère polaire dont l'huile de paraffine est dépourvue. La netteté et la reproductibilité des séparations observées avec ce type de phase stationnaire nous a incités, par la suite, à étendre leur emploi à la chromatographie sur papier des esters 3,5-dinitrobenzoïques des alcools gras, et des diesters 3,5-dinitrobenzoïques des polyéthylèneglycols. Nous réservons à une date ultérieure la publication de nos travaux à ce sujet.

Pour ce qui concerne la chromatographie selon BORECKÝ ET GASPARIČ, sur papier imprégné de formamide, des diesters 3,5-dinitrobenzoïques des polyéthylèneglycols, nous rapportons ici, d'une part, l'étude des facteurs susceptibles d'être responsables des irrégularités signalées plus haut, et d'autre part, la recherche de phases mobiles permettant de réaliser des séparations plus nettes et plus régulières.

Enfin, nous écartant de la méthode de BORECKÝ ET GASPARIČ, nous examinons la possibilité d'employer des supports de la phase stationnaire (formamide) autres que le papier (Kieselguhr en couche mince, colonne d'alumine).

Auparavant, nous tenons toutefois à préciser ici le mode opératoire utilisé par nous pour la préparation des esters 3,5-dinitrobenzoïques.

PRÉPARATION DES DIESTERS DES PEG

La préparation en série des esters dinitrobenzoïques peut être effectuée selon la méthode simple indiquée par BORECKÝ ET GASPARIČ[1]. Cependant, pour l'élimination de l'excès de chlorure de 3,5-dinitrobenzoyle, nous avons été conduits, d'une part, à substituer la soude 5 % au carbonate de sodium trop peu actif (préconisé par BORECKÝ ET GASPARIČ[1]), et d'autre part, à effectuer un lavage systématique des solutions benzéniques.

Nous opérons actuellement de la manière suivante:

Dans un tube à essais de 160 mm de longueur et 16 mm de diamètre, on pèse exactement 0.1 g de produit à étudier, et approximativement une quantité de Cl-DNB correspondant à un excès de 50 % par rapport à la quantité théorique nécessaire pour obtenir les diesters dans le cas des PEG. On mélange le tout avec une baguette de verre, et porte 20 min à 110°.

Après refroidissement, on ajoute d'abord 2 ml de benzène, puis 3–4 ml de soude à 5 %; on agite avec la baguette de verre pour dissoudre le produit de réaction, et l'on ajoute encore 8 ml de benzène de manière à obtenir une solution à 1 % en produit mis en oeuvre.

Le tube est bouché au moyen d'un bouchon de liège protégé par une feuille souple de polyéthylène, et agité mécaniquement pendant 5 min. On laisse décanter; la solution alcaline sous-jacente est pipetée, remplacée par 5 ml de soude à 5 % et le tube est soumis pendant 15 min à l'agitation mécanique. L'opération est répétée trois fois. On lave, de la même manière, à l'eau distillée jusqu'à neutralité (3–4 fois).

La phase benzénique restante est additionnée de sulfate de sodium anhydre et agitée afin de la sécher. On laisse reposer. La solution benzénique surnageante des dinitrobenzoates est alors prête à être déposée sur le papier pour la chromatographie.

Nous avons opéré en chromatographie descendante; les indications et les observations du texte qui suit sont relatives à ce mode opératoire. Le papier utilisé était le papier Arches 302. Ajoutons que la position verticale adoptée lors du développe-

ment correspondait au "sens machine" du papier; le déplacement de la phase mobile s'effectuait donc suivant le sens d'orientation des fibres.

Il convient peut-être de souligner que la présente étude de séparation chromatographique ne concerne que *les esters* 3,5-*dinitrobenzoïques* des classes de corps citées (polyéthylèneglycols). Si donc, dans le texte qui suit, il nous arrive d'utiliser des expressions telles que par exemple premiers termes, termes légers, termes lourds, etc., ce n'est que dans le dessein d'alléger le texte, et il faut toujours entendre par là les esters 3,5-dinitrobenzoïques de ces termes.

RÉVÉLATION DES TACHES

Nous avons mentionné que l'excès de la phase stationnaire (formamide) gênait la révélation des DNB sur le papier. Nous avons donc recherché tout d'abord un moyen d'y remédier.

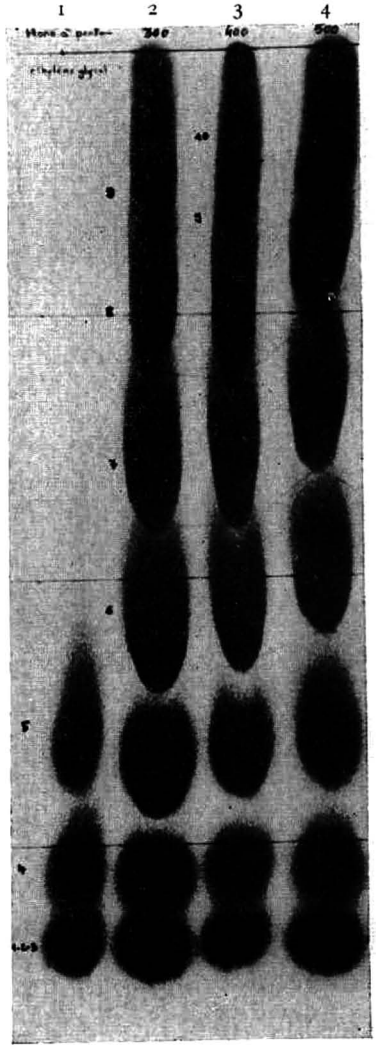


Fig. 1. Bis-3,5-DNB de polyéthylèneglycols techniques. 1 = mélange synthétique des mono-, di-, tri-, tétra- et pentaéthylèneglycols; 2, 3, 4 = polyéthylèneglycols techniques 300, 400, 500. Phase stationnaire: formamide (20% dans acétone). Phase mobile: hexane–benzène (1:1) (en vol.). Atmosphère de l'enceinte saturée avec hexane–benzene (1:1). Durée de développement: 6 h. Température: 17–19°.

Nous n'avons pas trouvé de solvant permettant, par lavage des chromatogrammes, d'éliminer le formamide sans dissoudre les DNB. Mais il est facile de s'en débarrasser en le volatilisant par chauffage des chromatogrammes à 75–80° pendant 1.5–2 h. Nous avons vérifié que, pendant ce traitement, les DNB ne se décomposent pas et ne diffusent pas dans le papier. Par suite du départ du formamide, la révélation ultérieure des taches se trouve grandement améliorée (intensité, sensibilité, stabilité accrues, Fig. 1).

Après révélation des DNB, les bases de Schiff formées (jaunes) ne sont plus solubles dans l'éther éthylique, ni même dans des mélanges éther–méthanol suffisamment riches en éther, ce qui permet, à ce stade, de laver les chromatogrammes et d'éliminer ainsi la majeure partie de l'excès des réactifs ayant servi à la révélation ($SnCl_2$, $SnCl_4$, HCl, *p*-diméthylaminobenzaldéhyde), et même le formamide ayant éventuellement échappé au chauffage. Ce traitement assure une meilleure conservation des chromatogrammes.

SYSTÈME FORMAMIDE/HEXANE–BENZÈNE

De même que BORECKÝ ET GASPARIČ[1], nous avons observé un accroissement notable des R_F, en fonction de la concentration, dans les dépôts, des termes correspondants, accroissement sensible surtout à partir du bis-3,5-DNB du pentaéthylèneglycol.

D'autre part, et également à partir du bis-3,5-DNB du pentaéthylèneglycol, les taches prennent une forme allongée particulière: tête de forme plus ou moins aiguë (en V, en W, parfois plus arrondie) où la concentration est maximale, suivie d'une queue élargie et diffuse, de concentration décroissante; cette queue bifurque même souvent en deux branches. Le phénomène, parfois très prononcé, gêne la séparation des DNB: les taches se recouvrent, souvent sur une partie importante de leur surface, et cela surtout pour les termes supérieurs (par exemple, Figs. 1 et 2)*.

A cet allongement des taches s'ajoute un élargissement par diffusion dans le papier au cours du développement, lequel diminue encore la netteté des séparations, et semble en même temps accentuer l'allongement des taches.

Enfin, malgré tous les soins apportés, une reproductibilité suffisante de la qualité des séparations reste difficile à atteindre.

Nous avons considéré un certain nombre de facteurs qui nous paraissaient susceptibles d'être responsables des phénomènes d'irrégularité observés.

Sauf indication contraire, les systèmes de phases utilisés dans l'étude de ces facteurs sont les suivants:

Phase stationnaire	*Phase mobile*
Papier imprégné avec une solution de formamide à 20%	Hexane–benzène (1:1)
Papier imprégné avec une solution de formamide à 25%	Hexane–benzène (3:4)

* Il est d'ailleurs probable que l'accroissement des R_F en fonction de la concentration des termes correspondants et l'allongement des taches sont imputables à une même cause: nous verrons en effet que l'importance des deux phénomènes augmente ou diminue simultanément.

Influence de la qualité du formamide

Des essais comparatifs de formamides d'origines et de puretés différentes (produit Prolabo tel quel, ou purifié par nos soins, produit Merck pur pour chromatographie) n'ont pas fait ressortir une influence significative du facteur pureté du formamide sur la qualité des chromatogrammes.

L'addition de petites quantités de produits de décomposition que l'on peut trouver dans le formamide (ammoniac, formiate d'ammonium, acide formique) n'a pas non plus paru exercer une influence notable.

Influence du solvant du formamide

Nous utilisions primitivement le méthanol comme solvant du formamide, pour l'imprégnation du papier. Mais nous avons constaté que les solutions méthanoliques de formamide se décomposaient rapidement, avec dégagement d'ammoniac ($\sim$ 24 h). Nous avons alors remplacé avantageusement le méthanol par l'acétone qui permet de réaliser des solutions beaucoup plus stables, pouvant être conservées plusieurs jours sans décomposition apparente. Les auteurs tchèques utilisent l'éthanol[1].

Influence de l'humidité

On effectue l'imprégnation du papier en formamide par immersion dans une solution de ce dernier dans un solvant volatil. Lors de l'évaporation du solvant suivant les indications de BORECKÝ ET GASPARIČ[1,2], c'est-à-dire dans l'atmosphère et à la température ambiante, il se produit inévitablement une absorption d'humidité par le papier imprégné, due à l'action conjuguée des propriétés hygroscopiques du formamide et du refroidissement provoqué par l'évaporation du solvant.

Nous avons cherché à éliminer l'influence de cette humidification en séchant, après départ du solvant, le papier imprégné de formamide dans une enceinte légèrement chauffée: par exemple, un séjour de 15 min dans une étuve, réglée à 45° et en présence de $CaCl_2$ anhydre, s'est avéré suffisant, sans provoquer de départ sensible de formamide. (Notons que ce traitement permet en même temps d'éliminer des traces de solvant, capables, le cas échéant, d'exercer une action perturbatrice (Réf. 2, p. 483).)

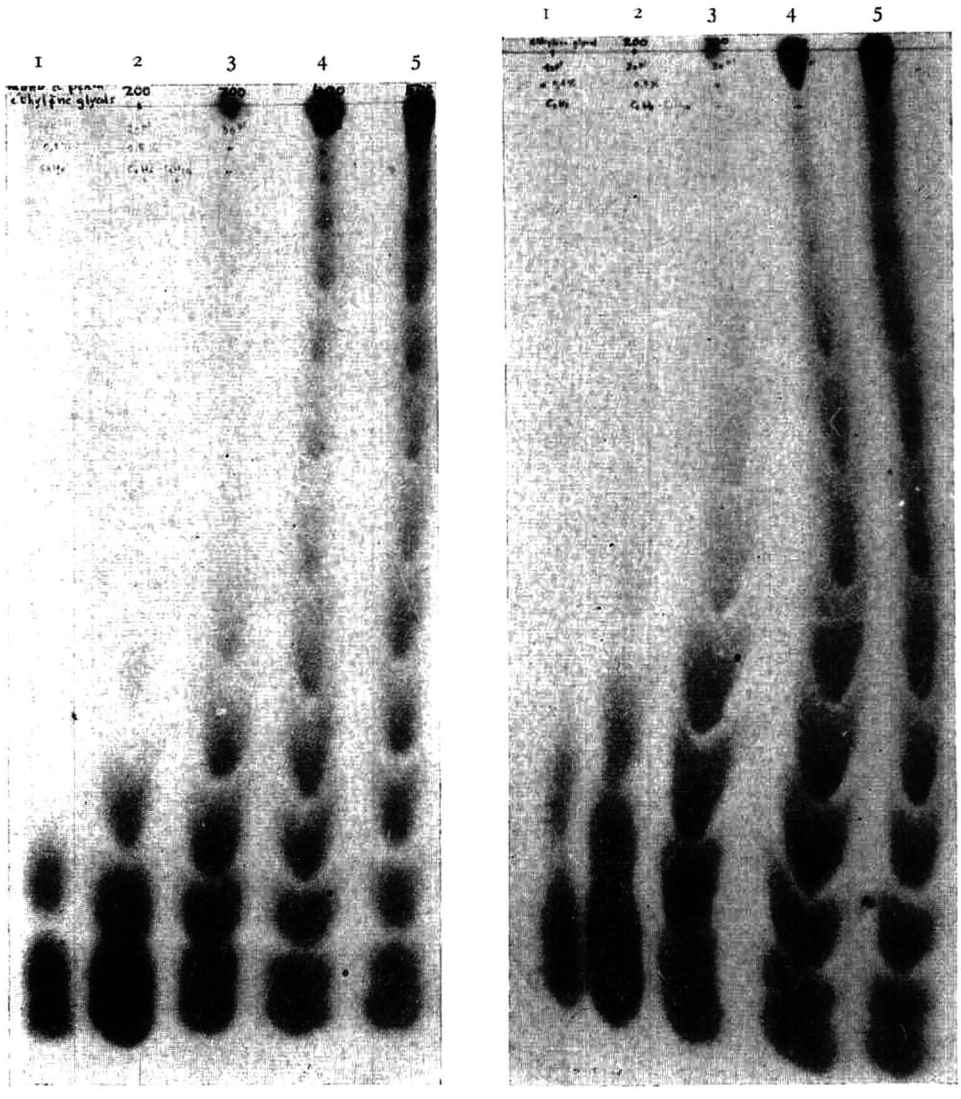
Après le dépôt des substances à chromatographier, on sèche à nouveau pendant 10 min dans les mêmes conditions.

Nous prenons également soin de dessécher l'atmosphère de la cuve de développement au moyen de $CaCl_2$ anhydre.

Dans ces conditions, nous avons constaté que l'effet d'élargissement diffus des taches se trouvait réduit notablement (Fig. 2a et 2b).

Influence du mode de dépôt des substances à chromatographier

Nous avons déjà dit plus haut (p. 212) que, dans le système considéré ici (formamide 20 ou 25 %/hexane–benzène (1:1) ou (3:4)), les R_F variaient en fonction directe de la quantité de substance déposée par unité de surface (concentration de substance sur le papier). Cette variation est accompagnée du phénomène d'allongement des taches. Nous nous sommes alors demandé si la forme elle-même des taches (forme en V ou W), de son côté, n'était pas à mettre en relation avec des variations de concentration en substance à l'intérieur de la surface de dépôt.



(a) (b)

Fig. 2. Bis-3,5-DNB de polyéthylèneglycols techniques. (a) Papier séché 15 min à 45° après imprégnation. (b) Papier non séché après imprégnation. 1 = mélange synthétique des mono-, di,- tri-, tétra- et pentaéthylèneglycols; 2,3,4,5 = polyéthylèneglycols techniques 200, 300, 400, 500. Phase stationnaire: formamide (20% dans acétone). Phase mobile: hexane–benzène (1:1). Durée de développement: 3 h 30 min. Température: ~ 18°.

Nous déposons les DNB des polyéthylèneglycols sous forme de leurs solutions dans le benzène. Si l'on effectue le dépôt en appliquant la pointe de la micropipette au point choisi sur le papier, il se produit autour de ce point une tache circulaire, constituant la surface de dépôt. Le diamètre de cette tache dépend du volume de solution déposée et de la rapidité d'évaporation du solvant (température, courants

d'air, etc.). Or, le benzène est, vis-à-vis des DNB, un éluant fort; il aura tendance à les entraîner vers la périphérie de la surface de dépôt.

On conçoit que le mouvement de la phase mobile à travers une surface de dépôt ainsi constituée (surface circulaire à concentration périphérique plus grande) ait tendance, étant donné l'influence sur les R_F de la concentration en substances, à donner des taches à double maximum (forme en W plus ou moins prononcé).

La réalisation d'une surface de dépôt plus homogène, obtenue par exemple en multipliant les touches à la micropipette et en les répartissant sur une surface rectangulaire, a permis d'obtenir des taches en forme de trapèzes plus ramassés.

Mais aucun artifice ne nous a permis, dans le système considéré ici, de supprimer ou de réduire l'allongement des taches proprement dit.

Influence de la saturation de l'atmosphère de la chambre de développement

Dans nos essais, l'atmosphère de la chambre de développement est saturée en phase mobile d'une part grâce à un bac, contenant celle-ci, placé au fond de la chambre, d'autre part grâce à une feuille de papier supplémentaire imbibée de phase mobile, dispensée par une augette spéciale située à la partie supérieure de l'enceinte.

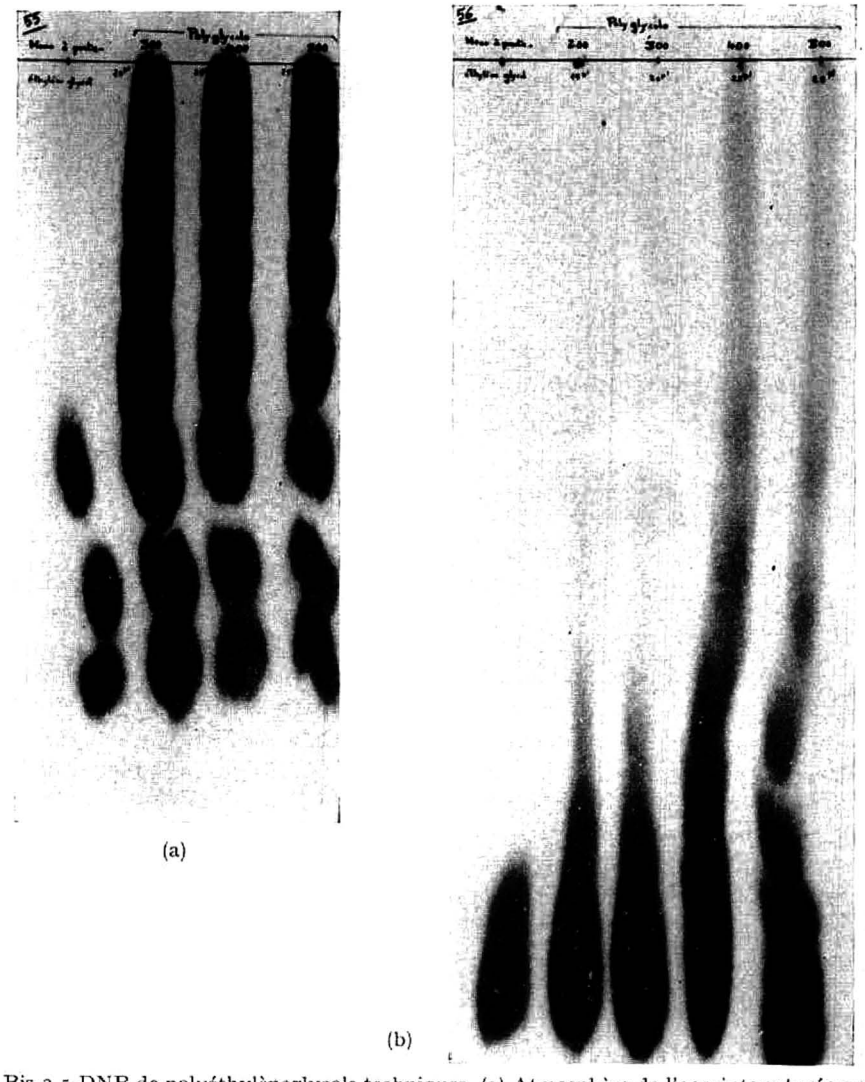
La saturation exacte de l'atmosphère de la chambre en phase mobile (hexane–benzène) est très importante, car elle influe directement sur la composition de la phase mobile parcourant la feuille de papier. Cette variation de composition, à coup sûr, perturbe le développement; elle pourrait très bien être responsable de l'allongement des taches: en effet, une saturation imparfaite semble devoir a priori entraîner un enrichissement, sur le papier, de la phase mobile en benzène, par suite d'une évaporation plus rapide de l'hexane. Et le benzène, éluant fort vis-à-vis des DNB, a tendance à les entraîner vers le front du liquide.

Effectivement, des essais en atmosphère saturée par un mélange hexane–benzène plus riche en benzène que la phase mobile, ont conduit à des chromatogrammes dont les taches sont très allongées; en même temps, l'on constate une augmentation générale des R_F (Fig. 3b). Inversement, les chromatogrammes obtenus en opérant en atmosphère saturée par un mélange plus riche en hexane que la phase mobile ont montré une atténuation sensible de l'allongement des taches, en même temps qu'une diminution des R_F (Fig. 3a).

Nous avons, également, réalisé volontairement une saturation imparfaite de l'enceinte, de manière à en vérifier directement l'influence, envisagée ci-dessus, sur la forme des taches. On constate là aussi une forte accentuation de l'allongement des taches, accompagnée d'une forte augmentation des R_F (mesurés au front des taches).

Ces essais vérifient donc bien que l'influence perturbatrice d'une saturation imparfaite de l'atmosphère de l'enceinte consiste en une accentuation de l'allongement, un étirement des taches.

Cependant, ce facteur ne peut pas être considéré comme une cause essentielle du phénomène pour la raison suivante: nous avons effectué des essais dans une atmosphère à la saturation et à l'homogénéité de laquelle avaient été apportés des soins tout particuliers (notamment grâce à un brassage de l'atmosphère de l'enceinte par ventilateur[3]: la saturation est vérifiée par le fait que la feuille de papier destinée à saturer l'atmosphère ne sèche plus, même à proximité du courant de vapeurs produit par le ventilateur). Et pourtant, le phénomène d'allongement des taches est demeuré toujours aussi prononcé.



(a) (b)

Fig. 3. Bis-3,5-DNB de polyéthylèneglycols techniques. (a) Atmosphère de l'enceinte saturée avec hexane–benzène (2:1). (b) Atmosphère de l'enceinte saturée avec hexane–benzène (1:2). Phase stationnaire: formamide (20% dans acétone); papier séché à 45° après imprégnation. Phase mobile: hexane–benzène (1:1).

Il reste que, lorsqu'il intervient, le facteur saturation imparfaite de l'atmosphère, joint d'autre part aux fluctuations de la concentration du papier en formamide[1,2] peut du moins rendre compte du défaut de reproductibilité observé dans les séparations.

Influence de la concentration du papier en phase mobile

Nous avons pensé aux perturbations que peut apporter à un développement régulier le fait que, au cours de la migration de la phase mobile, il existe sur le papier une diminution continue de la concentration en phase mobile depuis la ligne d'immersion jusqu'au front de progression.

Ce phénomène, qui est connu (*cf.*, entre autres, Réfs. 4, 5), est facilement observable dans le cas présent: la partie de la feuille de papier voisine de la ligne d'immersion prend un aspect mouillé alors que les régions proches du front apparaissent presque sèches, à tel point que le front de déplacement de la phase mobile devient de moins en moins discernable avec l'augmentation de la distance parcourue (ceci étant vrai même en atmosphère bien saturée).

Plus particulièrement, nous nous sommes demandé comment évoluerait le développement si l'on évitait, ou si l'on supprimait la zone très mouillée. Une première manière était d'éloigner de la ligne d'immersion les points de dépôt des produits à chromatographier (c'est d'ailleurs cette raison qui sans doute justifie la recommandation faite par certains auteurs de déposer les substances à une distance d'au moins 5 cm de la ligne d'immersion).

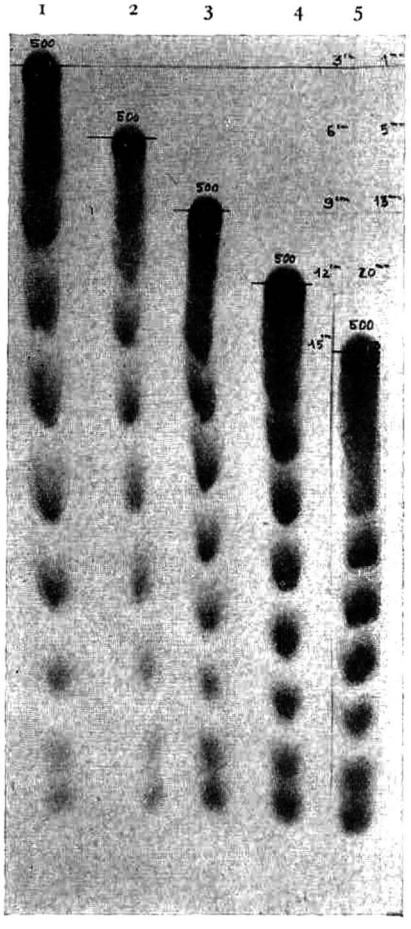


Fig. 4. Bis-3,5-DNB d'un polyéthylèneglycol 500 technique. Dépôt de la substance à des distances croissantes de la ligne d'immersion. Col. 1 = 3 cm; col. 2 = 6 cm; col. 3 = 9 cm; col. 4 = 12 cm; col. 5 = 15 cm. Phase stationnaire: formamide (20% dans acétone); papier séché à 45° après imprégnation. Phase mobile: hexane–benzène (3:4). Atmosphère saturée avec hexane–benzène (3:4). Température: 21–22°.

Une seconde manière consistait à abaisser le régime des tensions de vapeur dans l'atmosphère de l'enceinte par création dans celle-ci d'un gradient de température, de manière à diminuer, par évaporation, la concentration en phase mobile sur le papier: ce que nous avons tenté de réaliser par introduction de glace carbonique dans l'enceinte. C'est, en quelque sorte, prendre le contrepied de ce que nous avons recherché p. 215, mais d'une manière tout à fait particulière*.

(*a*) *Eloignement des points de dépôt des produits à chromatographier.* La Fig. 4 montre très nettement une diminution progressive de l'allongement des taches au fur et à mesure que l'on augmente la distance des dépôts à la ligne d'immersion. Pour une distance de 12–15 cm, les taches ne sont pratiquement plus allongées; la résolution est sensiblement améliorée.

(*b*) *Introduction de glace carbonique dans l'enceinte en début de développement.* Voici comment nous avons procédé: la saturation de l'atmosphère en phase mobile étant assurée selon les modalités indiquées plus haut, et le papier, portant les substances à chromatographier, étant en place dans son augette vide, on introduit rapidement dans l'enceinte 500–800 g de glace carbonique pilée; on ferme l'enceinte, puis on verse rapidement, par un orifice prévu à cet effet, la phase mobile dans son augette.

On observe alors, autour de la glace carbonique, l'apparition d'aiguilles de benzène et une condensation d'hexane (moins apparente). Entre temps, la progression de la phase mobile a commencé; mais, après une certaine avance (qui dépend des conditions opératoires; Fig. 5), le développement est pratiquement stoppé du fait de l'appauvrissement du papier en phase mobile. Puis, au fur et à mesure que disparaît la glace carbonique les conditions initiales se rétablissent, et le développement reprend progressivement.

Le résultat obtenu est intéressant: après révélation, on voit apparaître des taches presque rondes pour les termes inférieurs, beaucoup moins allongées pour les termes plus élevés, et la séparation est améliorée (Fig. 5, col. 1).

(*c*) *Combinaison des modifications* (*a*) *et* (*b*). Le chromatogramme de la Fig. 5 (col. 2–8) permet de voir que la combinaison des deux modifications (a) et (b) est encore plus efficace que chacune d'elles prise isolément: là encore les taches sont d'autant plus rondes et régulières que le point de dépôt a été éloigné davantage de la ligne d'immersion, et leur séparation devient cette fois pratiquement complète (alors que, notons-le en passant, les quantités de substances présentes dans les taches sont relativement très importantes).

Influence de la concentration du papier en phase stationnaire

Nous venons d'exposer que la diminution de la concentration en phase mobile sur le papier (diminution obtenue soit par introduction de glace carbonique dans l'enceinte, soit par l'artifice de l'éloignement des points de dépôt, soit encore par la combinaison des deux procédés) avait pour conséquence l'obtention de taches arrondies bien ramassées.

On peut se demander si le même effet ne pourrait pas être atteint par une voie inverse, c'est-à-dire par augmentation de la concentration du papier en phase stationnaire (formamide).

BORECKÝ ET GASPARIČ[1] imprègnent le papier au moyen d'une solution de for-

* Une technique analogue de création d'un gradient de température a déjà été décrite (DRAPRON[3]).

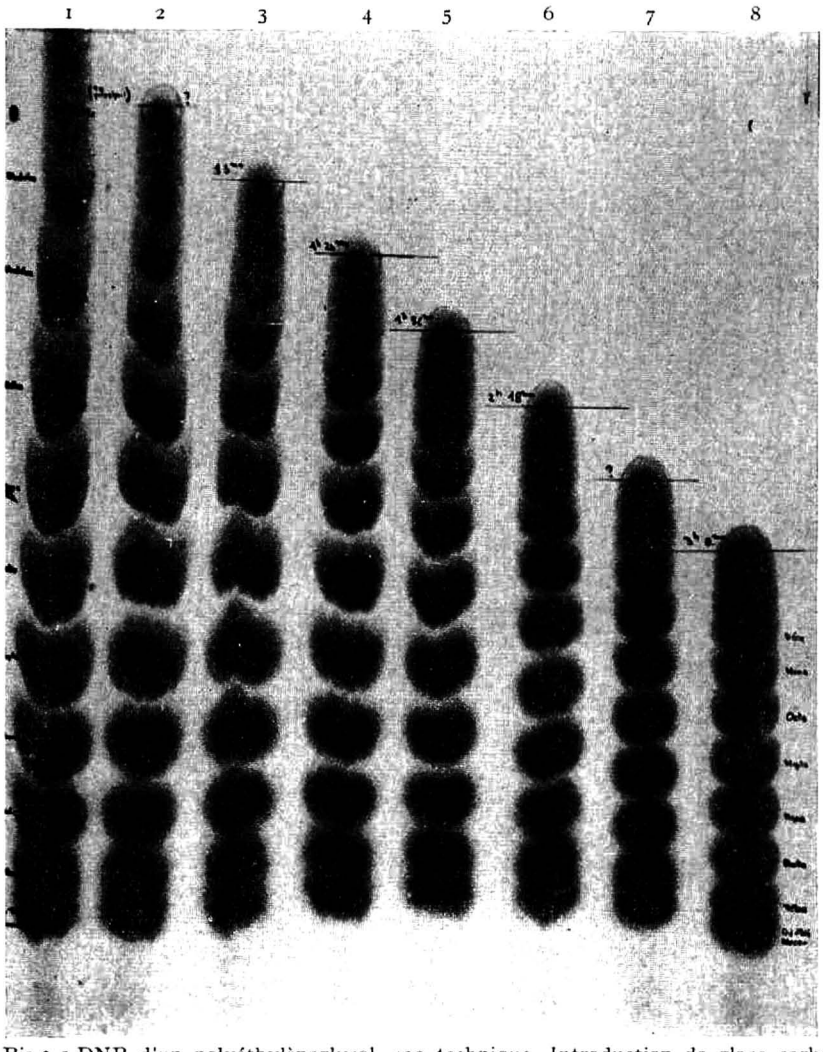


Fig. 5. Bis-3,5-DNB d'un polyéthylèneglycol 400 technique. Introduction de glace carbonique dans l'enceinte au début du développement. Dépôt de la substance à des distances croissantes de la ligne d'immersion. Col. 1 = 3 cm; col. 2 = 6 cm; col. 3 = 9 cm; col. 4 = 12 cm; col. 5 = 15 cm; col. 6 = 18 cm; col. 7 = 21 cm; col. 8 = 24 cm. Phase stationnaire: formamide (20 % dans acétone) papier séché à 45° après imprégnation. Phase mobile: hexane–benzène (1:1)

mamide à 20 % (dans l'éthanol). Nous avons alors imprégné le papier avec des solutions de formamide à 40 % et davantage (dans l'acétone).

Cependant, conformément à la formule:

$$R_F = \frac{1}{1 + \frac{C_s}{C_m}\frac{A_s}{A_m}}$$

où C_s/C_m = coefficient de partage entre phase stationnaire et phase mobile, A_s = aire de la section transversale de la phase stationnaire, A_m = aire de la section transversale de la phase mobile, conformément, aussi, aux observations faites par GASPARIČ ET

BORECKÝ[2] et par nousmêmes à ce sujet, l'augmentation de la concentration du papier en phase stationnaire a pour effet de diminuer les valeurs des R_F. Afin de compenser cette influence défavorable, nous avons été amenés à augmenter la proportion d'éluant fort (benzène) dans la phase mobile et à utiliser ainsi des mélanges hexane–benzène dont les proportions respectives étaient, par exemple, de 1:2.

Il convient de souligner que, pour obtenir des résultats satisfaisants, tant du point de vue de la qualité des séparations que de celui de la qualité de la révélation des chromatogrammes, avec des concentrations aussi élevées en formamide sur le papier, il est nécessaire de prendre les précautions indiquées plus haut: (a) séchage, avant développement, du papier imprégné en formamide (p. 213), (b) élimination totale de la phase stationnaire (formamide) avant révélation (p. 212).

Il est possible que les effets défavorables du formamide, constatés en l'absence de ces précautions, aient amené BORECKÝ ET GASPARIČ à ne pas dépasser la concentration de 20 % en formamide, pour la solution d'imprégnation.

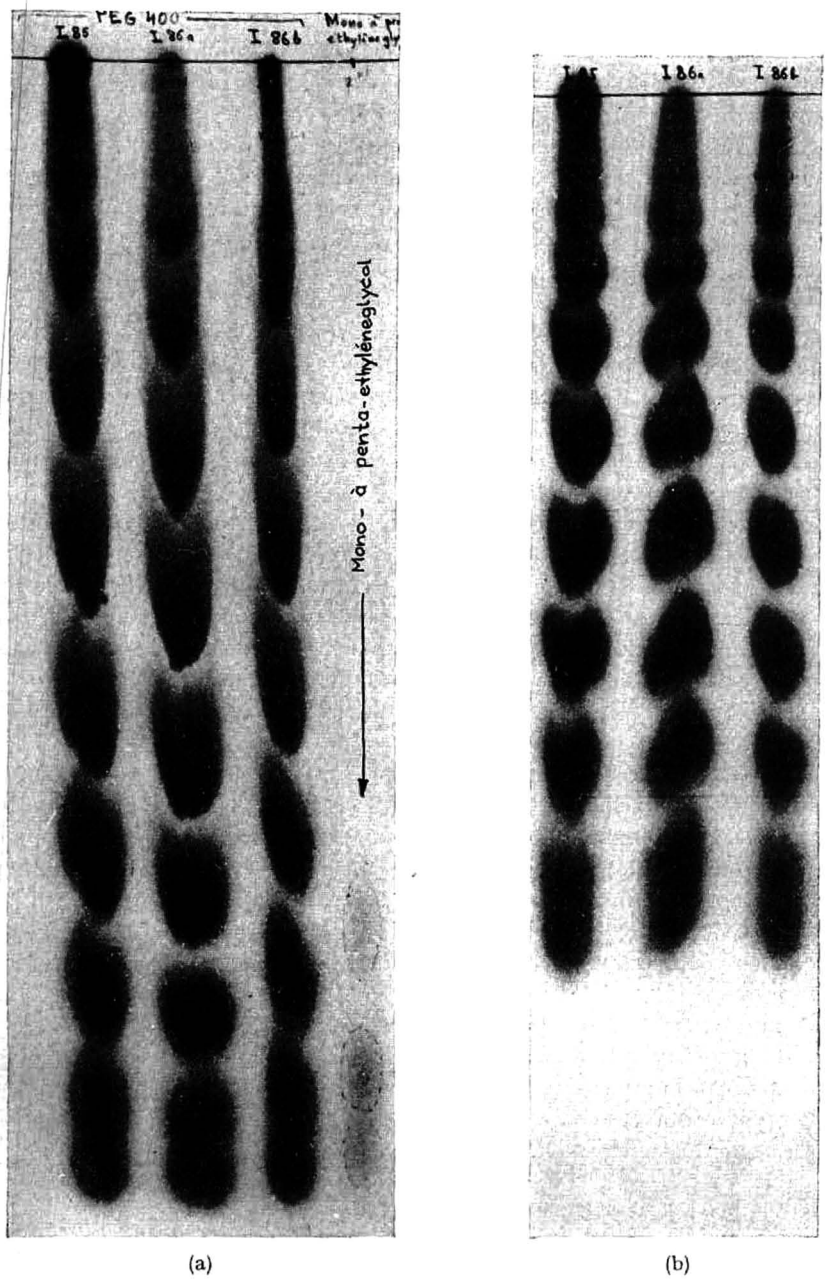
Afin d'observer l'action d'une concentration plus grande du papier en formamide, nous avons donc développé deux chromatogrammes simultanément, dans la même enceinte, l'un ayant été imprégné avec une solution de formamide à 20 % (dans l'acétone), l'autre avec une solution à 40 % (dans le même solvant). Les autres conditions opératoires étaient maintenues aussi identiques que possible: procédure d'imprégnation, séchage à 45° (selon mode opératoire p. 213), dépôts à la même distance de la ligne d'immersion (~ 3 cm), composition de la phase mobile hexane–benzène (1:2), saturation de l'atmosphère de l'enceinte, dispositif d'alimentation en phase mobile, température, etc.

Les résultats sont démonstratifs: sur le papier traité avec la solution de formamide à 40 % (et malgré la faible distance, 3 cm, des dépôts à la ligne d'immersion), on constate l'absence de tout allongement des taches (Fig. 6b). Au contraire, le chromatogramme réalisé sur papier traité au formamide à 20 % a conservé l'aspect habituel: taches allongées, qui se recouvrent partiellement (Fig. 6a).

Ces résultats (disparition de l'allongement des taches par augmentation de la concentration du papier en phase stationnaire), joints à ceux du paragraphe précédent (disparition de l'allongement des taches par diminution de la concentration du papier en phase mobile), font donc apparaître un lien, nécessaire à l'obtention de taches régulières, entre quantités de phase stationnaire et de phase mobile en présence sur le papier.

Ceci nous amène à reconsidérer la manière de préparer le papier imprégné en phase stationnaire. En effet, jusqu'ici, dans un souci de régularité d'imprégnation, nous avions, à l'encontre de BORECKÝ ET GASPARIČ, suspendu le papier imprégné, aux fins d'égouttage et d'évaporation, de telle manière que la future ligne de départ fût placée en haut. BORECKÝ ET GASPARIČ placent, dans cette opération, la ligne de départ en bas (Réf. 2, p. 482). La concentration du papier en phase stationnaire est alors plus élevée à la ligne de départ, et décroit lorsque l'on s'en éloigne (Réf. 2, p. 481)*. De cette manière, le gradient de concentration du papier en phase station-

* On pourrait même, à ce propos, objecter au raisonnement de la p. 218 (influence de l'éloignement des points de dépôt) que l'effet observé est dû à ce gradient (de sens croissant) de concentration du papier en phase stationnaire, et non pas au gradient (de sens décroissant) de concentration du papier en phase mobile. Mais, nous l'avons vérifié, la variation de la concentration de la phase stationnaire le long du papier, sur une longueur de 10–15 cm, n'est pas assez importante pour produire un effet aussi notable.



(a) (b)

Fig. 6. Bis-3,5-DNB d'un polyéthylèneglycol 400 technique. (a) Papier imprégné avec solution de formamide à 20%. (b) Papier imprégné avec solution de formamide à 40%. Papier séché à 45° après imprégnation. Phase mobile: hexane–benzène (1:2). Atmosphère de l'enceinte saturée avec hexane–benzène (1:2), et brassée par un ventilateur. Durée d'élution: 5 h 15 min. Température: 19°.

naire est de même sens que le gradient de concentration du papier en phase mobile qui s'établit lors du développement. Ce mode opératoire est donc plus indiqué dans le cas étudié ici, puisqu'il tend à corriger partiellement le déséquilibre, sur le papier, entre quantités de phase stationnaire et de phase mobile en présence. Nous l'avons utilisé pour effectuer le chromatogramme de la Fig. 7, où l'on peut voir en effet une certaine diminution de l'allongement, bien que la résolution soit ici incomplète. Avec ce mode opératoire, il suffit de porter la concentration de la solution d'imprégnation à 30 ou 35 % (au lieu des 40 % indiqués plus haut) pour obtenir de bons résultats.

Influence de la concentration du papier imprégné en substance déposée

Le fait que l'augmentation de la concentration du papier en phase stationnaire a pour effet de supprimer l'allongement des taches conduit à se demander s'il n'intervenait pas, lors de l'emploi de papier traité avec des solutions à 20 % seulement en phase stationnaire (mode opératoire de BORECKÝ ET GASPARIČ[1,2]), un phénomène de saturation de celle-ci en substance à chromatographier, saturation que l'augmentation de la quantité de phase stationnaire ferait cesser.

S'il y a saturation, on peut aussi bien la faire cesser en diminuant la concentration en substance dans les taches déposées. Nous avons par conséquent étudié l'influence de la variation de cette concentration sur la forme des taches et sur la résolution. A cet effet, nous avons effectué, dans le système formamide à 20 %/hexane–benzène (4.5:5.5), la chromatographie d'une série de dépôts de même volume (50 μl), donc de même surface, de solutions benzéniques du même produit de dinitrobenzoylation d'un polyéthylèneglycol 400 technique, à des concentrations variables: 0.1 %, 0.2 %, 0.5 %, 1 % et 2 %.

On peut alors constater (Fig. 7) que, en effet, l'allongement des taches diminue et que la résolution s'améliore avec la diminution de concentration des solutions déposées. L'effet est cependant beaucoup moins important que l'on pourrait s'y attendre: pour voir disparaître l'allongement des taches, il faut descendre jusqu'à une concentration de 0.1 %, concentration dix fois plus faible que celles que nous avons utilisées en général, et avec lesquelles nous avons cependant obtenu des taches non allongées et bien séparées, simplement en doublant la concentration du papier en phase stationnaire.

Le phénomène paraît donc plus complexe qu'une simple question de saturation de la phase stationnaire en produit à chromatographier. Cependant, la saturation, comme toujours en chromatographie, fixe une limite supérieure, à ne pas dépasser, pour la concentration en substance déposée du papier imprégné, limite qui, naturellement, varie avec le degré d'imprégnation.

Conclusion

Il semble donc bien, d'après les essais décrits ci-dessus, que l'allongement des taches soit dû essentiellement à un déséquilibre entre quantité de phase stationnaire et quantité de phase mobile en présence sur le papier. L'équilibre, par contre, paraît rétabli aussi bien lorsque (toutes autres modifications mises à part) l'on diminue, par un moyen approprié, la concentration du papier en phase mobile, que lorsque l'on augmente la concentration du papier en phase stationnaire.

Nos essais semblent, en outre, mettre en évidence une influence, sur l'allonge-

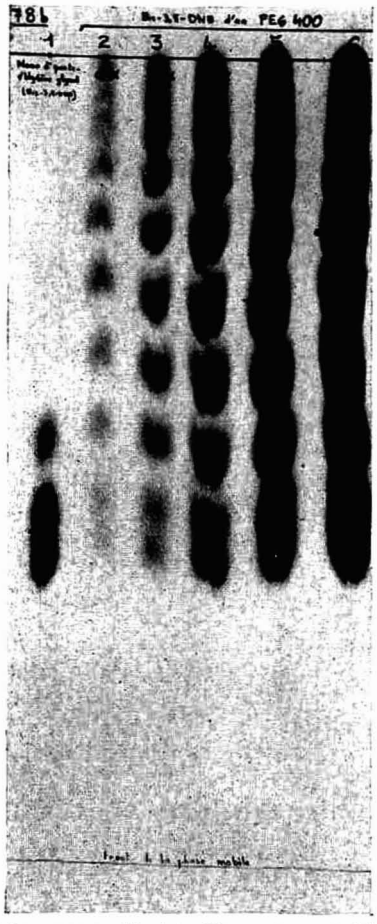


Fig. 7. Col. 1 = bis-3,5-DNB des mono-, di-, tri-, tétra- et pentaéthylèneglycols; col. 2,3,4,5,6 = bis-3,5-DNB d'un polyéthylèneglycol 400 technique à 0.1, 0.2, 0.5, 1 et 2 % (pourcentages exprimés en PEG mis en oeuvre). Phase stationnaire: formamide à 20 % (dans acétone); égouttage ligne de départ en bas; papier séché à 45° après imprégnation. Phase mobile: hexane–benzène (4.5:5.5). Durée d'élution: 2 h 55 min. Température: 19°.

ment des taches, de la concentration en substance à chromatographier dans la surface de dépôt; en tout état de cause, et pour un système donné il faut éviter des concentrations trop élevées; la limite à ne pas dépasser, pour les concentrations des solutions à déposer, dans le système formamide 20 % se situe à 0.1 ou 0.2 % et dans le système formamide 40 % à 1 ou 2 % (avec le papier Arches 302).

Nous avons pu dégager également l'influence d'autres facteurs sur la qualité des chromatogrammes, ce sont:

(i) L'humidité (élargissement diffus des taches).

(ii) Le défaut de saturation de l'atmosphère de l'enceinte (enrichissement, sur le papier, de la phase mobile en benzène, d'où augmentation des R_F et étirement généralisé des taches).

(iii) Le mode de dépôt de la substance à chromatographier (hétérogénéité de la surface de dépôt).

En résumé, les améliorations introduites dans la procédure de BORECKÝ ET GASPARIČ sont de deux sortes: celles qui concernent le développement des chromatogrammes, celles qui concernent leur révélation.

Les premières comprennent:

(a) L'alternative: augmentation de la concentration du papier en phase stationnaire, "diminution" (voir p. 216-218) de la concentration du papier en phase mobile.

(b) La détermination des limites supérieures de la concentration des surfaces de dépôt en produit à chromatographier, en fonction du système de phases utilisé.

(c) L'élimination de l'humidité par séchage du papier imprégné en phase stationnaire, à 45° en présence de $CaCl_2$ anhydre.

(d) La saturation et l'homogénéisation de l'atmosphère de l'enceinte par brassage.

(e) La réalisation de surfaces de dépôt (des substances à chromatographier) plus homogènes en concentration.

Les secondes (voir p. 212) sont les suivantes:

(a) Élimination de la phase stationnaire (formamide) *avant révélation* (traitement thermique à 75–80°).

(b) Élimination des réactifs excédentaires après révélation, par lavage des chromatogrammes (par mélange éther éthylique–méthanol).

L'ÉTHER ÉTHYLIQUE COMME PHASE MOBILE

Nous avons essayé d'autres phases mobiles (heptane–benzène, heptane–toluène, etc.). Si la séparation des termes légers se montrait meilleure (différences de R_F plus marquées), celle des termes lourds était par contre moins bonne. Par conséquent, nous n'avons pas retenu ces phases mobiles.

Mais nous avons obtenu des séparations d'une qualité excellente en utilisant comme phase mobile l'éther éthylique. La résolution est particulièrement nette, comme le montre, par exemple, le chromatogramme de la Fig. 8, obtenu avec les dérivés bis-3,5-dinitrobenzoylés des premiers représentants de la série, purs (mono-, di-, tri-, tétra- et pentaéthylèneglycols), et avec les dérivés bis-3,5-dinitrobenzoylés de polyéthylèneglycols techniques de P.M. moyens compris entre 200 et 600:

(i) Les taches sont rondes, séparées complètement les unes des autres, jusqu'au dixième terme.

(ii) Les R_F des constituants se montrent relativement indépendants de leur concentration dans les dépôts; par conséquent, les identifications sont plus aisées et plus sûres.

(iii) Alors qu'avec le mélange hexane–benzène comme phase mobile, les trois premiers termes ne sont pas séparés, seuls les deux premiers ne sont pas résolus en utilisant l'éther éthylique comme phase mobile.

Il est nécessaire de saturer l'éther en formamide, celui-ci étant légèrement soluble dans l'éther. La volatilité de l'éther éthylique oblige à assurer une étanchéité suffisante de l'enceinte, d'abord pour des raisons de régularité de développement, ensuite, pour des raisons de sécurité. De même, la manipulation, après développement, des papiers imbibés d'éther éthylique exige l'observation de certaines précautions, ce qui est aisément concevable. D'ailleurs, les mêmes servitudes valent, encore davantage peut-être, pour l'emploi de solvants tels que benzène, hexane, etc. Toutefois, le fait

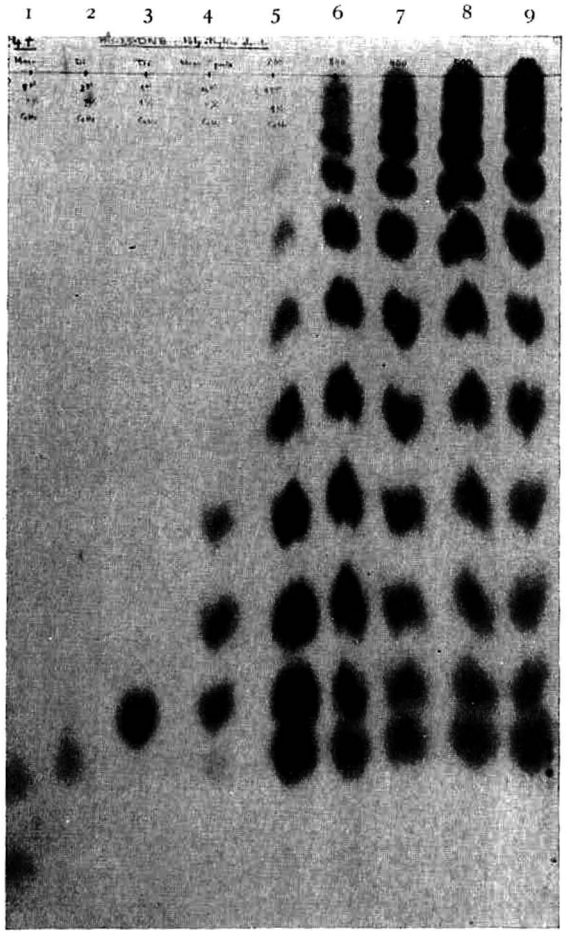


Fig. 8. Bis-3,5-DNB de polyéthylèneglycols techniques. 1 = glycol tache supérieure (tache inférieure non identifiée); 2 = diéthylèneglycol; 3 = triéthylèneglycol; 4 = mélange synthétique des mono-, di-, tri-, tétra- et pentaéthylèneglycols (mono et di en faible proportion); 5 = PEG 200; 6 = PEG 300; 7 = PEG 400; 8 = PEG 500; 9 = PEG 600. Phase stationnaire: formamide (20% dans acétone); papier séché à 45° après imprégnation. Phase mobile: éther éthylique saturé de formamide. Durée de développement: 3 h. Température: 17–18°.

que la phase mobile soit constituée par un solvant unique constitue, en soi, une notable simplification, puisque tout risque de variation dans la composition de la phase mobile, pendant la durée du développement, se trouve écarté; l'obtention aisée de meilleurs résultats doit sans aucun doute être attribuée pour une part à cet avantage.

La manifestation simultanée de la disparition complète de l'allongement des taches et de l'insensibilité relative des R_F des constituants vis-à-vis de leur concentration dans les dépôts, confirme à nos yeux l'existence d'un lien entre ces deux phénomènes.

La netteté particulière des séparations obtenues avec ce système est très favorable à une étude quantitative de la composition des polyéthylèneglycols techniques, étude qu'il est dans notre intention d'entreprendre.

UTILISATION DE SUPPORTS DE LA PHASE STATIONNAIRE AUTRES QUE LE PAPIER

Dans la chromatographie sur papier imprégné, le rôle du papier paraît être simplement celui de support de la phase stationnaire. Il est, d'ailleurs, sans doute souhaitable, en chromatographie de partage, que le rôle du support soit aussi minime que possible.

Nous avons donc pensé qu'il était possible d'obtenir, par exemple sur couche mince ou sur colonne, imprégnées de formamide, les mêmes résultats que sur papier imprégné.

En effet, nous avons obtenu les mêmes séparations (des neuf à dix premiers termes des bis-3,5-DNB des PEG) et d'une qualité égale, sur couche mince de Kieselguhr imprégnée de formamide (au moyen d'une solution à 20 % dans l'acétone). Comme liant de la couche mince, plutôt que le plâtre très fragile, nous avons utilisé la gomme du Sénégal, laquelle donne un revêtement très adhérent et très résistant. Là aussi, la phase mobile éther éthylique saturé de formamide donne de bonnes séparations (taches rondes complètement séparées), tandis que les mélanges hexane–benzène donnent des taches allongées en V se recouvrant partiellement. On observe seulement une rétention un peu plus grande des DNB (R_F plus faibles). La révélation est effectuée de la même manière que sur papier (pulvérisation de chlorure stanneux, puis de *p*-diméthylaminobenzaldéhyde) et les précautions prises (séchage à 45° après imprégnation en particulier) sont les mêmes.

Des essais sur colonne (par exemple alumine imprégnée de formamide) ont également permis d'obtenir la séparation des premiers termes des diesters 3,5-dinitrobenzoïques des polyéthylèneglycols (depuis le quatrième jusqu'au neuvième) en quantités pondérales (20–30 mg de chacun des constituants ont été ainsi obtenus). L'éluant utilisé était le mélange hexane–benzène, la quantité de benzène étant progressivement accrue au fur et à mesure de l'élution.

La progression des zones dans la colonne était suivie d'une part par leur pouvoir absorbant en lumière U.V. réfléchie, et d'autre part par pesée des fractions éluées (le formamide est insoluble dans les mélanges hexane–benzène). Cette méthode a toutefois comme inconvénient la lenteur d'élution des taches.

REMERCIEMENTS

Nous tenons à exprimer ici nos remerciements à la Direction Technique de la Société Française des Matières Colorantes, dans les services de laquelle nos travaux ont été effectués et qui a bien voulu nous autoriser à publier cette étude, à Monsieur R. GOUPIL, pour les conseils dont nous avons bénéficié, et à Monsieur N. DEVILLE dont nous avons pu apprécier l'aide technique.

RÉSUMÉ

L'étude analytique des polyéthylèneglycols par chromatographie sur papier de leurs diesters 3,5-dinitrobenzoïques, telle qu'elle a été proposée par BORECKÝ ET GASPARIČ[1], conduit à des résultats très intéressants. Cependant, certaines irrégularités (allongement des taches, diffusion) réduisent la netteté des séparations. Il a donc été procédé à une étude des causes de ces perturbations, ainsi qu'à la recherche d'autres phases mobiles, permettant d'obtenir une netteté et une reproductibilité plus grandes dans les séparations.

Cette étude a montré que la cause essentielle de perturbation (formation de taches allongées se recouvrant partiellement) pouvait être attribuée à un déséquilibre entre quantité de phase stationnaire (formamide) et quantité de phase mobile (mélange hexane–benzène), en présence sur le papier. Un autre facteur, l'humidité présente dans le papier imprégné, ajoute ses effets au premier (taches diffuses).

Une phase mobile, l'éther éthylique, en association avec le formamide comme phase stationnaire, s'est montrée particulièrement intéressante: les taches obtenues sont presque rondes et leur séparation est complète.

Des supports autres que le papier pour la phase stationnaire (formamide) ont également été examinés. L'utilisation de Kieselguhr en couche mince, lié à la gomme du Sénégal, et imprégné de formamide, a donné des séparations de qualité équivalente à celles obtenues sur papier imprégné. De même, la chromatographie sur colonne d'alumine imprégnée de formamide a permis la séparation de quantités pondérales des neuf premiers termes.

SUMMARY

Analysis of polyethylene glycols by paper chromatography of their bis-3,5-dinitrobenzoates according to the method of BORECKÝ AND GASPARIČ, gives useful results. However, some irregularities (elongation of the spots, diffusion) make the separations less distinct. Therefore the causes of these disturbances were studied and a search was made for other mobile phases with which better separations and greater reprodicibility might be obtained.

This investigation showed that the main cause of the disturbances (formation of elongated spots, partially overlapping) was a lack of equilibrium between the amount of stationary phase (formamide) and the amount of mobile phase (hexane–benzene mixture) present on the paper. Another factor, the moisture of the impregnated paper, has an additional effect (diffuse spots).

One mobile phase, ethyl ether used in conjunction with formamide as stationary phase, is particularly interesting, since the spots obtained are practically round and completely separated from each other.

Other supports for the stationary phase (formamide) besides paper were also examined. Thin layers of kieselguhr with Senegal gum as binding agent and impregnated with formamide, gave separations of the same quality as those obtained with impregnated paper. Chromatography on columns of alumina likewise made it possible to separate weighable amounts of the first nine members of the series.

BIBLIOGRAPHIE

1 J. BORECKÝ ET J. GASPARIČ, *Mikrochim. Acta*, (1961) 96.
2 J. GASPARIČ ET J. BORECKÝ, *J. Chromatog.*, 5 (1961) 466.
3 R. DRAPRON, Séance du 20.1.1961 de la Section de Chimie analytique de la Soc. Chim. de France. Résumé: *Bull. Soc. Chim. France*, (1961) 447.
4 J. C. GIDDINGS, G. H. STEWART ET A. L. RUOFF, *J. Chromatog.*, 3 (1960) 239.
5 S. E. WOOD ET H. H. STRAIN, *Anal. Chem.*, 26 (1954) 260.

DETECTION OF NON-REDUCING CARBOHYDRATE COMPOUNDS WITH COMPLEX CUPRATES(III)

J. KOCOUREK, M. TICHÁ AND J. KOŠTÍŘ

Department of Biochemistry, Charles University, Prague (Czechoslovakia) *

AND

L. JENŠOVSKÝ

Institute of Physics of Solids, Czechoslovak Academy of Sciences, Prague (Czechoslovakia)

(Received August 15th, 1963)

INTRODUCTION

Owing to the anomalous valency state of the central copper atom the complexes of trivalent copper act as powerful oxidizing agents, especially in an alkaline medium. This property of the complex bound copper(III) was used for the first time by BECK[1] as a semi-quantitative analytical method for the oxidative degradation of proteins. By far the most useful application of these complex copper(III) salts is to the oxidation of polyhydroxy compounds. This reaction can be followed electrometrically or spectrophotometrically and has been used *e.g.* in the quantitative estimation of tartrates[2] or for the detection of sugars and their derivatives on paper chromatograms[3]. The potassium periodatocuprate(III) reagent used by BONNER[3] for this purpose has in the complex anion the ligand of another strong oxidizing agent, namely periodate. It seemed interesting to investigate the detection of carbohydrates and other organic compounds by means of the telluratocuprate(III) anion $[Cu(TeO_6)]^{9-}$, where the only oxidizing agent is the trivalent copper. The different oxidizing properties associated with the two complex ions were considered as a possible means of distinguishing between the structures of various compounds. This means of identification proved unsuccessful and in fact on comparing our results with those of BONNER[3], no qualitative difference could be found in the action of periodatocuprates(III) and telluratocuprates(III). The new reagent, however, is worth investigating for its high sensitivity to a wide range of organic compounds, *e.g.* the various non-reducing carbohydrates dealt with in this paper.

EXPERIMENTAL

Reagent

The 0.05 *M* telluratocuprate(III) solution was prepared as described by JENŠOVSKÝ[4-6] and BONNER[3], using the equivalent amount of potassium tellurate instead of potassium periodate.

Sample solutions

The samples were dissolved in ethyl alcohol in concentrations of 0.1 % w/v.

* Albertov 2030, Praha 2 — Nové Město, Czechoslovakia.

Paper chromatography

The descending technique was employed, using Whatman No. 1 or No. 3 filter paper in a *n*-butanol–acetic acid–water (10:1:3) system, except in the case of the higher oligosaccharides, where the system *n*-propanol–ammonia–water (6:3:1) was used.

Detection of the spots

The paper chromatograms were thoroughly dried and, when free from all acid residues, were sprayed with the reagent. The sugar compounds give white spots on a distinctive deep yellow-brown background. The background disappears after a few minutes but a permanent record of the spots can be made either by marking with pencil or by spraying the chromatogram, immediately after the maximum contrast of the spots has been reached, with a solution of rosaniline as described by BONNER[3]. Red spots on a greenish yellow background were then obtained. A second method for stabilizing the chromatograms, especially useful where Whatman No. 3 paper was used, consisted in spraying with TTC* solution (2 % w/v sol. of TTC in water mixed with the same volume of 1 *N* NaOH) at 50–60° in the dark[7,8]. At the positions occupied by the oxidized compounds deep rosy spots appeared on a pink background. For the location of water insoluble carbohydrate derivatives, the standard dipping technique was found more suitable than spraying.

TABLE I

R_F VALUES OF NON-REDUCING FREE OLIGOSACCHARIDES, GLYCITOLS, GLYCOSIDES, GLYCOSANS AND GLYCONIC ACID LACTONE LOCATED BY MEANS OF TELLURATOCUPRATE(III) REAGENT WITH A SENSITIVITY OF 1 μg

Compound	*Solvent system*	R_F
Saccharose	*n*-BuOH–AcOH–H_2O (10:1:3)	0.08
Trehalose		0.55
Raffinose	*n*-PropOH–NH_4OH–H_2O (6:3:1)	0.48
Stachyose		0.41
D-Glucitol (Sorbitol)	*n*-BuOH–AcOH–H_2O (10:1:3)	0.05
1,5-Anhydro-D-glucitol		0.25
D-Mannitol		0.05
myo-Inositol		0.05
Methyl β-D-glucopyranoside	*n*-BuOH–AcOH–H_2O (10:1:3)	0.22
n-Propyl β-D-glucopyranoside		0.61
Isopropyl β-D-glucopyranoside		0.55
Isobutyl β-D-glucopyranoside		0.73
tert.-Butyl β-D-glucopyranoside		0.64
Cyclohexyl β-D-glucopyranoside		0.72
Phenyl β-D-glucopyranoside		0.55
m-Hydroxyphenyl β-D-glucopyranoside[a]		0.52
o-Hydroxymethylphenyl β-D-glucopyranoside (Salicin)		0.52
Phenyl β-cellobioside		0.35
1,6-Anhydro-β-D-glucopyranose	*n*-BuOH–AcOH–H_2O (10:1:3)	0.45
D-Galactonic-γ-lactone	*n*-BuOH–AcOH–H_2O (10:1:3)	0.21

[a] *m*-Hydroxyphenyl β-D-glucopyranoside gives, as all free phenols, a stable red-brown coloration without a further stabilizing spray.

* Triphenyltetrazolium chloride.

TABLE II

R_F VALUES OF VARIOUS SUBSTITUTED CARBOHYDRATE COMPOUNDS DETECTED BY MEANS OF TELLURATOCUPRATE(III) REAGENT WITH A SENSITIVITY OF 5 TO 10 μg
System *n*-butanol–acetic acid–water (10:1:3).

Compound	R_F
1,2,3,6,2′,3′,4′,6′-Octa-O-acetyl-β-maltose	1.0
3,4,6-Tri-O-acetyl-D-glucal	1.0
1,2-O-Isopropylidene-α-D-glucofuranose	0.70
1,2,5,6-Di-O-isopropylidene-α-D-glucofuranose[a]	0.98
1,2-O-Isopropylidene-5,6-anhydro-α-D-glucofuranose	0.95
1,6-Anhydro-2-O-*p*-tolyl-β-D-altropyranose[b]	0.85

[a] Does not react with the telluratocuprate(III) but develops a deep rosy coloration with the TTC reagent without any previous treatment. This remarkable reaction is difficult to explain as the compound is perfectly stable in alkaline medium and has one single hydroxyl group in the C-3 position.

[b] Sensitivity = 50 μg.

RESULTS

The miscellaneous carbohydrate compounds tested for the applicability of the telluratocuprate(III) reagent can be divided into three groups according to the sensitivity of the reagent.

(1) The unsubstituted sugars, glycosides, glycosans and anhydrosugars are detectable in amounts of 1 μg.

(2) In substituted sugars, *e.g.* in fully acylated sugars easily hydrolysable in the strong alkaline medium, or in various alkylidene derivatives with at least one pair of vicinal hydroxyl groups, the sensitivity varies between 5 and 10 μg. In some cases, *e.g.* 1,6-anhydro-2-O-*p*-tolyl-β-D-altropyranose, a compound with a single hydroxyl pair adjacent to the large *p*-tolyl group, the limit of sensitivity was shifted to 50 μg.

(3) The non-reactive substances include especially the alkali-stable polytopic alkylidene and arylidene derivatives such as the various di- and tri-benzylidene or isopropylidene derivatives of monoses and glycitols, *e.g.* 1,2,5,6-di-O-isopropylidene-α-D-glucofuranose.

The telluratocuprate(III) reagent has been used with success for the detection of sugar compounds in plant extracts and in mixtures of enzymic transglycosylation products. R_F values of carbohydrate compounds located with the reagent are summarized in Tables I and II.

DISCUSSION

The results summarized in this paper as compared with those of BONNER[3] indicate the interesting fact that in both periodatocuprates(III) and telluratocuprates(III) applied as locating reagents in paper chromatography the only oxidizing agent is the trivalent copper. The sensitivity of telluratocuprate(III) is in most cases even slightly higher than that of periodatocuprate(III) as, after spraying, a picture is developed in which white spots show up very vividly against a deep yellow-brown background.

SUMMARY

The use of potassium telluratocuprate(III) is described for the detection of non-reducing carbohydrate compounds on paper chromatograms. The sensitivity of the reagent is 1 μg to unsubstituted sugars and glycosides and 5 to 10 μg to a number of carboxylic acid esters and alkylidene carbohydrate derivatives tested.

REFERENCES

[1] G. BECK, *Mikrochemie ver. Mikrochim. Acta*, 35 (1951) 1, 152.
[2] L. JENŠOVSKÝ, *Collection Czech. Chem. Commun.*, 22 (1957) 313.
[3] T. G. BONNER, *Chem. & Ind. (London)*, 1960, 345.
[4] L. JENŠOVSKÝ, *Chem. Listy*, 50 (1956) 1956; *C. A.*, 50 (1956) 14438.
[5] L. JENŠOVSKÝ, *Z. Anorg. Allgem. Chem.*, 307 (1961) 208.
[6] L. JENŠOVSKÝ, *Z. Anorg. Allgem. Chem.*, 307 (1961) 219.
[7] K. WALLENFELS, *Naturwissenschaften*, 37 (1950) 491.
[8] W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444.

WATER CONTENT OF PAPER AS A VARIABLE IN PAPER CHROMATOGRAPHY

ARTHUR J. TOMISEK AND PAULA WEDELES ALLAN

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Ala. (U.S.A.)*

(Received September 12th, 1963)

INTRODUCTION

In our long use[1,2] of the common two-dimensional papergram system, phenol–water followed by butanol–propionic acid–water[3], for the separation of various soluble, intracellular metabolic products, we have experienced only rare lapses of reproducibility; and most of these lapses we had crudely associated with the season of the year. HANES and his co-workers reported in great detail on the effects and the methods of control of the moisture content of chromatographic paper[4–6]. These reports suggested to us that moisture control might prove useful even in chromatographic systems less sophisticated than those developed in the HANES laboratory. The present report describes the simple procedure of moisture control which we have found satisfactory over the past two years.

EXPERIMENTAL

General procedure

Whatman No. 1 paper was used, twelve $18^1/_4 \times 22^1/_2$ in. sheets per cabinet, for descending chromatography.

The first (short) dimension of the paper was developed with a phenol–water mixture (722:278, w/w) (cloud point, $18^1/_2$ to $20^1/_2°$) at the controlled room temperature of 21 to 22°. When made with distilled phenol[7] and stored in the dark, this solvent remains colorless for over a month. As suggested by WADE *et al.*[5], there was no preliminary equilibration of paper with phenolic solvent.

The second solvent consisted of *n*-butanol–water (1770:119, v/v) and propionic acid–water (88:110, v/v) mixed in equal volumes, the cloud point being $18^1/_2$ to 20°. Commercial grade propionic acid (E.I. du Pont Co.)** was used directly, without redistillation. An arbitrary one hour of equilibration of paper with this solvent was allowed, following any preadjustment of the paper's moisture content.

Moisture adjustment

For most of the chromatographic experiments reported here, papers were stored for 24 h in the chromatographic cabinet with three 15 cm petri dishes containing thin

* Affiliated with Sloan-Kettering Institute for Cancer Research, New York, N.Y.

** This material was stored for several years without any alteration of its miscibility in the solvent system. This stability was not characteristic of other commercial propionic acids (Cliffs Dow Chemical Co. and Celanese Chemical Co.) even after redistillation.

layers of various "constant humidity agents". These agents, together with the equilibrium relative humidities as measured on our instruments (see below) were: water (87 % R. H.); saturated aqueous ammonium sulfate, technical grade (72 % R.H.); sodium acid sulfate, technical grade crystals moistened with water (53 % R.H.); chromium trioxide, technical flakes moistened with water (42 % R.H.); potassium acetate, N.F. (24 % R.H.); and calcium chloride, anhydrous (10 % R.H.).

In experiments where equilibration time was a variable, the cabinet also contained a thermograph, and a very small fan (an 0.2 A tube-cooling fan available from radio supply stores). Ordinarily, we use this fan in conjunction with a proportionating timer (General Electric Co., TSA-14, 5-min cycle) set for 20 % on.

Relative humidity measurements were primarily by a humidigraph (Model 4033, The Bristol Co., Waterbury, Conn.), although a membrane hygrometer (Model 201, Serdex, Inc., Boston, Mass.) was included as a check and to provide a faster-responding instrument for short-term observations. Readings taken from a sling psychrometer (in the range of 25 to 75 % R.H.) were used to calibrate the humidigraph.

All instruments, the fan, and the dishes of humidity control agents were removed from the cabinet before the addition of chromatographic solvent.

RESULTS

Time required for equilibration

The relevance of humidigraph readings to water content of chromatographic paper was checked directly by using an external balance to weigh a pair of paper sheets suspended inside a cabinet containing ten other sheets and humidity control agent (potassium acetate or ammonium sulfate). Paper weights and humidigraph readings were both plotted against time, adjusting the graphic scales to cause coincidence of the curves at both their beginnings and their final equilibrium values (*e.g.*, Fig. 1). Under conditions of constant fan operation (except when weighing) the humidigraph responded somewhat faster than the paper; but in absence of fanning the paper responded somewhat faster. Discrepancy in the two responses never exceeded 8 units of % R.H. in either the humidifying or drying direction; and it was always 2 units or less by the time the paper weight had come to within 5 units of the final equilibrium value.

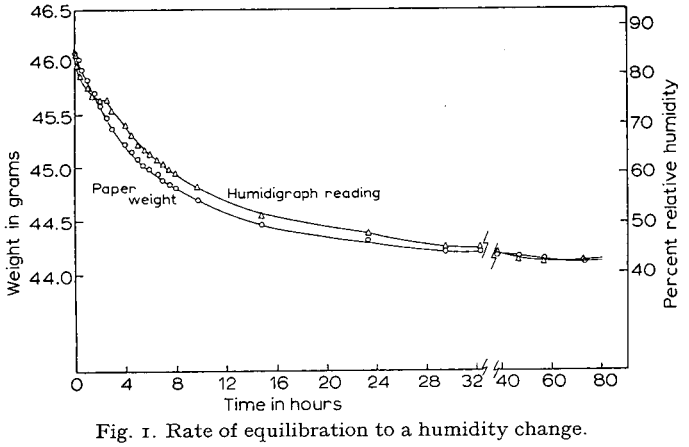


Fig. 1. Rate of equilibration to a humidity change.

Based on humidigraph readings only, using any of the humidity control agents*, 24 h were required for equilibration within 5 units of the final relative humidity. With the use of the circulating fan, this same extent of equilibration required only 9 h or 6 h, depending on whether the fan was used 20 % of the time or continuously. The use of intermittent fanning was devised primarily for insulated chromatographic boxes (Chromatocabs) in order to keep the temperature rise within the box from exceeding 0.5°.

These data have led to the evolution of the following routine procedure. When empty cabinets are available, the papers are stored in the cabinets with the sodium acid sulfate for at least 24 h. When cabinets are not available the papers are hung in the laboratory. Procedure with the latter papers varies according to the humidity of the laboratory. If the humidigraph shows that the relative humidity of the laboratory was within the chosen limits (50 to 75 % in our case) for the preceding 6 h, the papers may be placed in cabinets without any humidity control agent and stored there till ready for the addition of solvent. If the humidity record of the laboratory indicates the need for adjusting the moisture content of the papers, the papers are placed in a cabinet with an appropriate humidity control agent and a circulating fan.

Loss of equilibration upon opening of boxes

The literature on general chromatographic procedures leaves the impression that opening a box lid to permit the filling of solvent troughs (following equilibration of papers with solvent vapors) can cause a sufficient loss of solvent vapor to affect the chomatographic result. With regard to the special case of equilibration with water vapor, we find this impression to be misleading.

On days of especially low humidity we have tried opening boxes of high internal humidity (containing no papers) for various lengths of time. By comparison of the final equilibrium R.H. within the box to the initial and external R.H., we concluded that opening the box for 3 min permitted the interchange of only $^1/_3$ of the air volume of the box. To be sure, this did involve two precautions: the tray door near the bottom of the box was never opened when the top was open, and strong air currents were avoided by shutting off circulating fans in both room and box. What makes this result especially significant is the fact that the paper in a box contains several times the water content of the airspace in the box.

Effect of water content of paper on excursion values

For purposes of Figs. 2 and 3, sheets of known compounds were subjected to chromatography in only one dimension. The curves in Figs. 2 and 3 are chosen as typical. For both solvents a generality may be noted: excursion values are not appreciably affected by the water content of the paper, except that papers with very high water content produce a marked opening up of the chromatographic pattern near the origin together with some crowding in the remaining areas. Within this qualitative generality, some compounds differ markedly in the extent of their response to papers of very high water content. Glutamine in phenol–water solvent (Fig. 2) and lactic acid-^{14}C in butanol–propionic acid–water solvent (Fig. 3) illustrate that this quanti-

* After several days exposure to R.H. about 90 %, the humidigraph required several weeks to regain its former calibration.

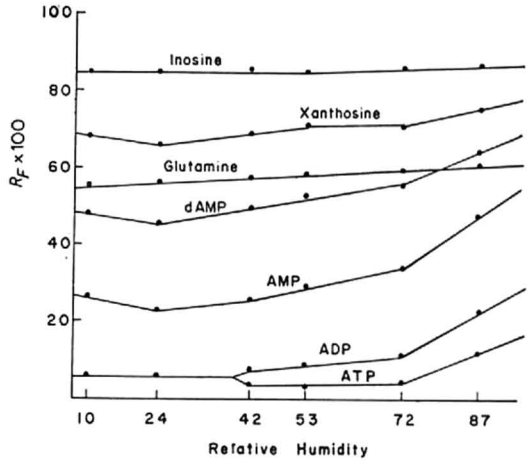


Fig. 2. Averaged excursion values of known compounds in phenol–water solvent. The merging of the ADP and ATP curves indicates that the compounds were no longer separated.

tative difference can result in a reversal of spot positions within the chromatographic pattern. Both of the above reversals had been observed on numerous occasions in our studies of mouse tissue and bacterial extracts before we resorted to controlling the water content of our papers. Additional reversals for the phenol–water solvent may be noted in Figs. 4 and 5 by comparing glutamic acid, aspartic acid, and malic acid with AMP, IMP, and ADP; also, (not illustrated) there is a similar reversal of glutamic acid and CMP. Indeed, it appears general for the phenol–water solvent, that the high moisture content of papers causes larger increases of R_F for purine and pyrimidine ribonucleotides than for aminoacids and hydroxyacids.

In the chromatography of larger amounts of tissue extracts, papers of low moisture content frequently lead to elongation of the fast-moving spots in the phenol–water direction. We take this to indicate that dry papers have less loading capacity.

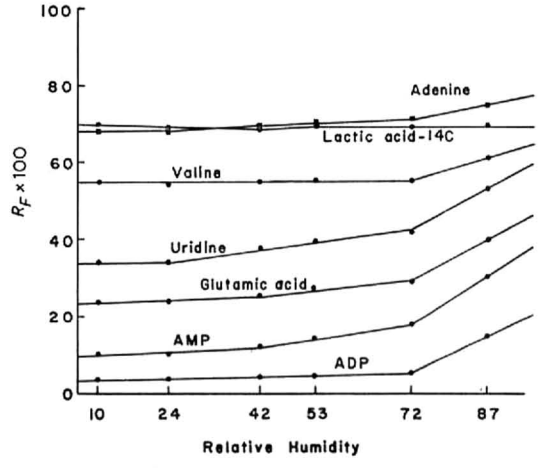


Fig. 3. Averaged excursion values of known compounds in butanol–propionic acid–water solvent.

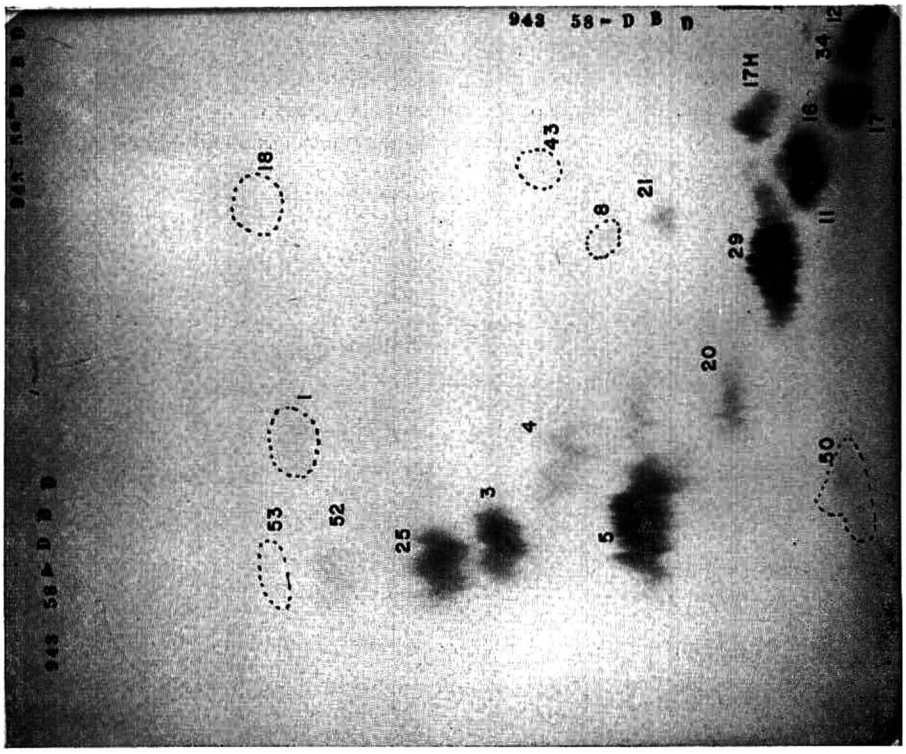


Fig. 4. Phenol–water solvent (right to left) on a paper equilibrated to 10% relative humidity; then butanol–propionic acid solvent (bottom to top) on paper equilibrated to 53% R.H. The photograph is from an autoradiogram after separation of the soluble fraction components from *Escherichia coli*[2] which had assimilated formate-^{14}C. Spot identies are as for Fig. 5.

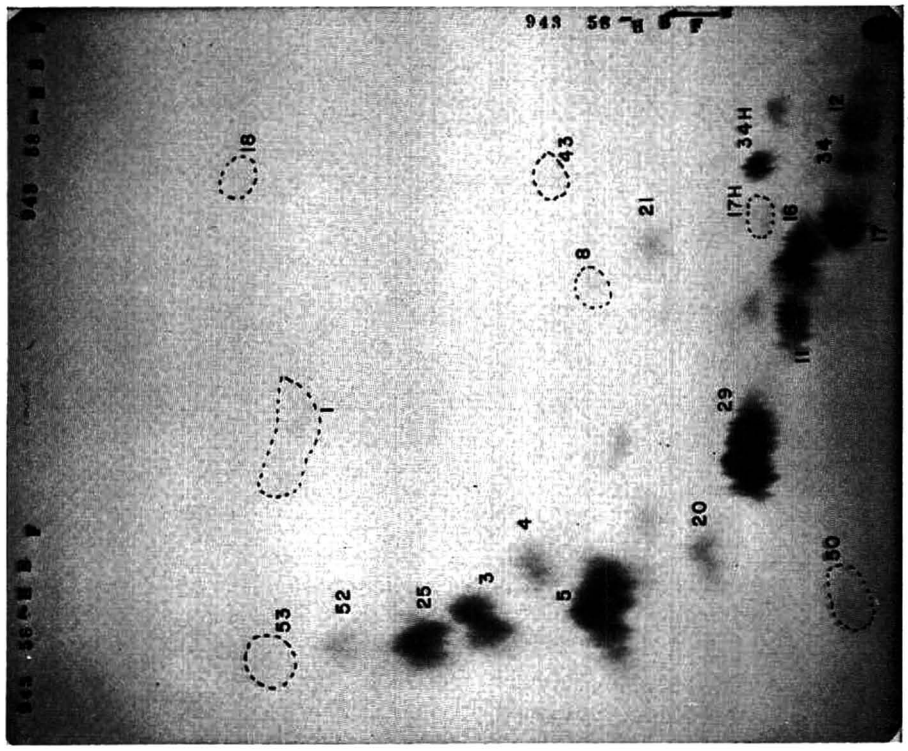


Fig. 5. Phenol–water solvent on a paper equilibrated to 87% R.H. Otherwise similar to Fig. 4. Identities of the spots are: 1, lactic acid; 3, hypoxanthine; 4, guanine; 5, inosine; 8, glutamic acid; 11, IMP; 12, GTP; 16, GMP; 17, ADP; 17H, hydrolytic artifact of ADP; 18, fumaric acid; 20, dAMP; 21, aspartic acid; 25, adenosine; 29, AMP; 34, ATP; 34H, hydrolytic artifact of ATP; 43, malic acid; 50, NAD; 52, adenine; 53, 5'-methylthioadenosine.

DISCUSSION

Our main purpose in controlling the water content of chromatographic paper is to insure reproducibility of chromatographic patterns. We use this chromatographic system in testing for possible effects of various biological inhibitors upon the intermediary metabolism of purines (or pyrimidines). In deciding whether preliminary experiments warrant a more complete study, it is essential that individual spots in the normal pattern must be identifiable by visual inspection. Toward this end, the given procedure for control of water content of paper has been very useful. This has been true even though our use of the procedure is not so much for precise control as it is for the avoiding of harmful extremes.

Another purpose in controlling water content of paper might be the deliberate use of extreme conditions to produce some specially desired effect. In our work, we have occasionally resorted to very moist papers to effect difficult separations in the ribonucleotide area.

The use of a humidity meter whose sensing element consists of wood fibres provides a reasonably close analog of the water content of paper. In addition, the recorded time course of humidity variation is more pertinent to the moisture content of paper than a single humidity reading on a rapidly-responding instrument.

ACKNOWLEDGEMENTS

This work was supported by grants from the Alfred P. Sloan Foundation and the Charles F. Kettering Foundation.

SUMMARY

A simple procedure is described for improving the reproducibility of two-dimensional papergrams made by the solvent systems: phenol–water followed by butanol–propionic acid–water. The procedure involves control of the water content of the chromatographic paper prior to the addition of solvent. Some effects noted upon the variation of the water content of paper are described for some chromatographic patterns of biochemical interest.

REFERENCES

[1] R. K. ALLISON, H. E. SKIPPER, M. R. REID, W. A. SHORT AND G. L. HOGAN, *J. Biol. Chem.*, 204 (1953) 197.
[2] A. J. TOMISEK AND M. R. REID, *J. Biol. Chem.*, 237 (1962) 807.
[3] A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS AND W. STEPKA, *J. Am. Chem. Soc.*, 72 (1950) 1710.
[4] C. S. HANES, *Can. J. Biochem. and Physiol.*, 39 (1961) 119.
[5] E. H. M. WADE, A. T. MATHESON AND C. S. HANES, *Can. J. Biochem. and Physiol.*, 39 (1961) 141.
[6] C. S. HANES, C. K. HARRIS, M. A. MOSCARELLO AND E. TIGANE, *Can. J. Biochem. and Physiol.*, 39 (1961) 163.
[7] O. J. DRAPER AND A. L. POLLARD, *Science*, 109 (1949) 448.

CHROMATOGRAPHY ON ION EXCHANGE PAPERS

XII. THE ADSORPTION OF METAL IONS ON VARIOUS ANION EXCHANGE PAPERS—FURTHER RESULTS

G. BAGLIANO, G. GRASSINI, M. LEDERER AND L. OSSICINI

Laboratorio di Cromatografia del C.N.R.
e Laboratorio di Chimica delle Radiazioni e Chimica Nucleare del C.N.E.N.,
Istituto di Chimica Generale ed Inorganica,
Rome (Italy)

(Received September 9th, 1963)

INTRODUCTION

The adsorption of numerous metal ions on anion exchange resin paper, DEAE cellulose paper, amino-ethyl cellulose paper and Whatman No. 1 paper from HCl solutions was described in a previous communication of this series[1]. The differences between the adsorption from HCl and LiCl were considered in a preliminary note[2] dealing only with two typical anions, ReO_4^- and $AuCl_4^-$. In this paper we shall describe further investigations on the adsorption of metal ions on various anion exchange papers which enlarge upon the problems already dealt with and add new data for analytical purposes.

The directions in which we extended the previous work were (i) obtaining data of adsorption from HCl solutions of ions which had not been previously studied [As(III), As(V), Ru(III) and U(IV)], (ii) studying Amberlite WB-2 weak anion exchange resin paper with a few typical metal ions, (iii) making an extensive survey of the adsorption of metal ions from LiCl solutions and (iv) studying the adsorption of a number of metal ions from HBr solutions.

The general technique of washing the papers and chromatographing in small jars by the ascending method was employed as described previously[1]. When additional operations were performed these are mentioned under the relevant sub-heading.

(i) Further data on the adsorption of metal ions from HCl

Fig. 1. shows the R_F values of As(III), As(V), U(IV) and Ru(III) plotted against the normality of HCl. The R_F values on the strong base resin paper (SB-2) agree well with the data of KRAUS AND NELSON[3]. The adsorption of Ru(III) (*i.e.* chlororuthenite) on aminoethylcellulose is much higher than may be expected from the adsorption on the DEAE cellulose and is difficult to explain by ion exchange and adsorption phenomena; the latter should be more pronounced for the DEAE paper.

(ii) The adsorption of some metal ions on the Amberlite weak anion exchange paper (WB-2) from HCl or LiCl

The weak base resin paper (WB-2) containing Amberlite IR-4B differs from the strong base resin paper SB-2 (containing Amberlite IRA-400) not only in the type of ion

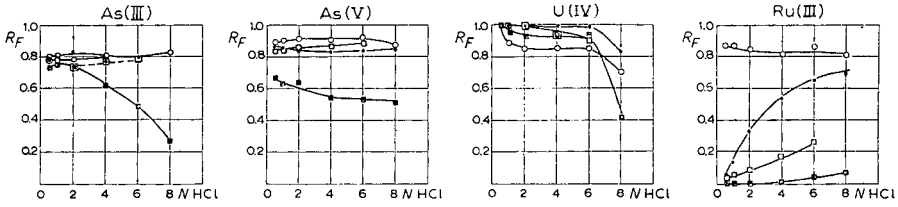


Fig. 1. R_F values of some metal ions plotted against the concentration of HCl. O—O on Whatman No. 1 paper; ■—■ on SB-2 strong base resin paper; ●—● on Whatman DE 20 DEAE paper; □—□ on Whatman AE 30 amino ethyl cellulose paper.

exchange groups but also in the type of polymer, as Amberlite IR-4B is a phenol-formaldehyde resin while Amberlite IRA-400 is a polystyrene resin. It is unlikely that in solutions containing more than 1 *N* HCl any difference in ionisation exists between the two types of exchange groups, the quaternary ammonium and the amino group. The effect of the substituents on the nitrogen as well as the differences in the polymer network should, however, both produce differences in the non-polar nature of the two resins. In Fig. 2 we have given the R_F values on WB-2 and SB-2 papers both with HCl and with LiCl (containing 1 *N* HCl) for a few metal ions. The adsorption on the WB-2 paper is consistently lower, the biggest differences occurring in those solutions where equilibria of neutral species seem to be involved *e.g.* with Fe(III) and UO_2(II) in intermediate concentrations of chloride ions. In the case of Cu(II) there seems to be little difference between the two resins. Strongly adsorbed

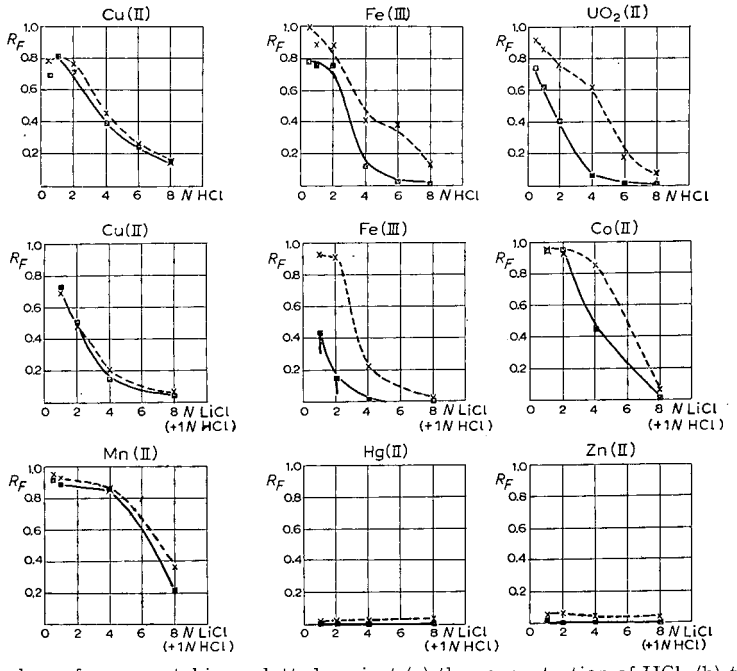
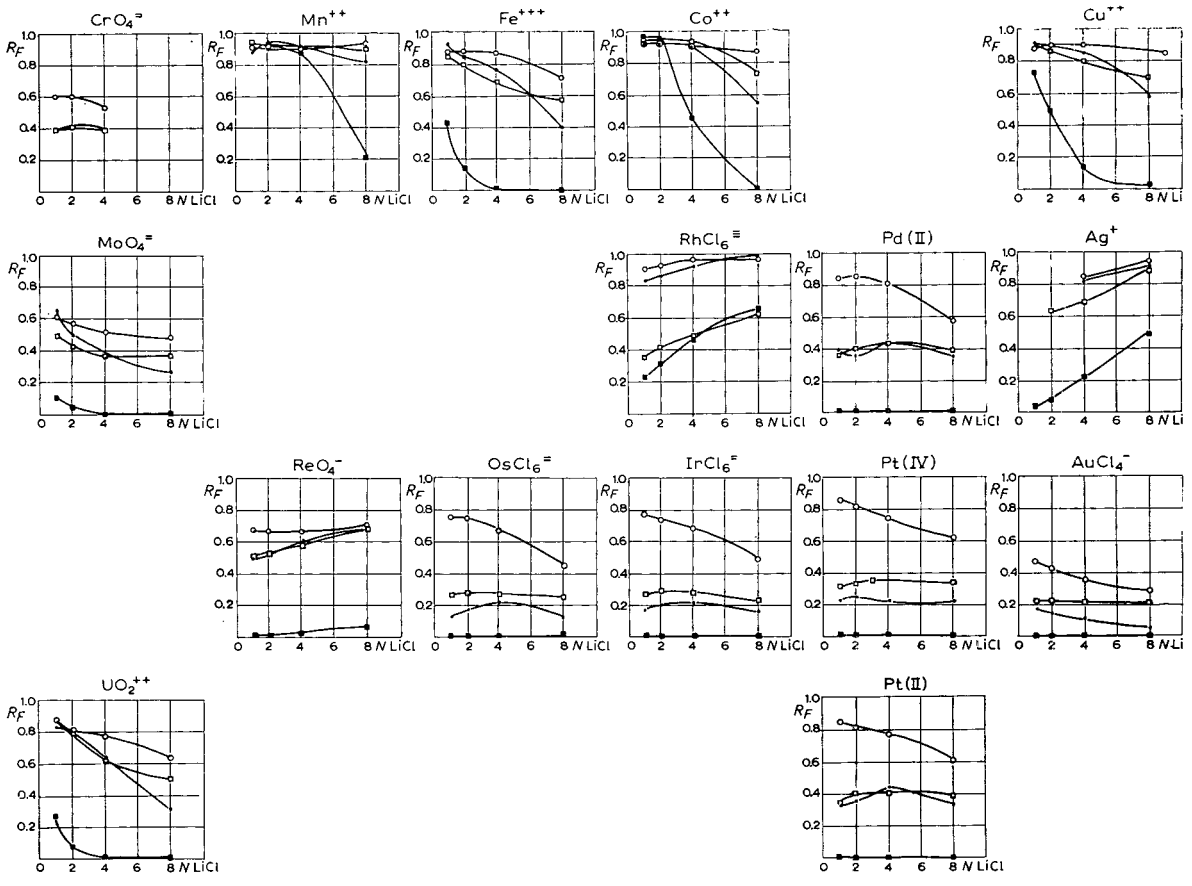


Fig. 2. R_F values of some metal ions plotted against (a) the concentration of HCl, (b) the concentration of LiCl (containing 1 *N* HCl throughout). ■—■ on SB-2 strong base resin paper; ×—× on WB-2 weak base resin paper.



anions such as $HgCl_4^{2-}$ and $ZnCl_4^{2-}$ have slightly higher R_F values on the weak base resin paper. The results obtained indicate that an extensive study of resins other than polystyrene resins may produce still more interesting differences in the sequence of separations. Cu(II) for example moves faster than Fe(III) on the SB-2 paper but more slowly on the WB-2 paper (up to 4 *N* LiCl).

(iii) A survey of the adsorption of metal ions from LiCl solutions

In preliminary work with LiCl[2] it was noted that if small amounts of HCl are added to LiCl (*e.g.* 0.1 *N* HCl) the H^+ front moves much more slowly than the liquid front. As this would have a considerable influence in work with some hydrolysable metal ions, we increased the HCl concentration to 1 *N* throughout in this work. The ions were chromatographed with solutions 1 *N* with respect to HCl and 1, 2, 4 and 8 *N* with respect to LiCl. The total chloride concentration in these cases is thus the sum of LiCl and HCl concentrations.

In Fig. 3, R_F values have been plotted against LiCl concentration for four papers (Whatman No. 1, Whatman DE 20, Whatman AE 30 and Amberlite SB-2 papers) and the ions are arranged according to their positions in the periodic table. The results

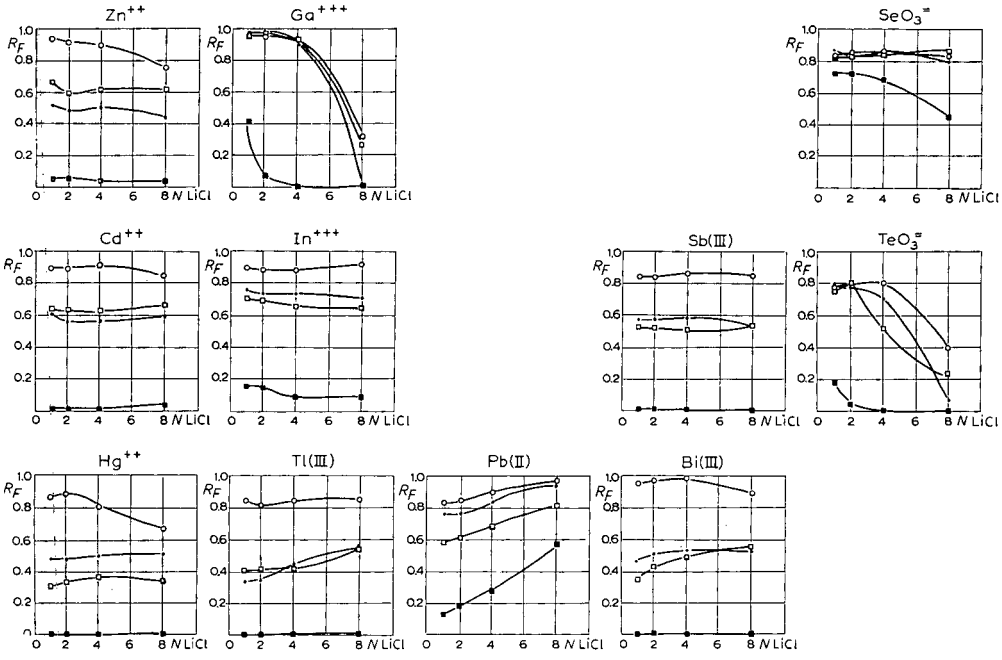


Fig. 3. R_F values of metal ions on various anion exchange papers plotted against the concentration of LiCl (containing 1 N HCl). Symbols as in Fig. 1.

with LiCl must be discussed by comparing them with the results with HCl published previously[1].

Ag^+ and Pb^{2+} have practically identical R_F values in HCl and LiCl. Here it should be remembered that these two ions seem to be retained almost entirely by ion exchange and not by adsorption. The results with LiCl seem to confirm these findings since in ion exchange the cation of the eluant should theoretically have no effect.

With the chloro-anions such as $IrCl_6^{2-}$, $PtCl_6^{2-}$, $AuCl_4^-$, $HgCl_4^{2-}$ and also with CrO_4^{2-} etc., there is a marked desorption with an increase in the HCl concentration on the cellulose exchangers. This is not the case (or to a much less extent) in LiCl, there being little variation of the R_F values. The salting-out effect previously observed[2] with $AuCl_4^-$ seems to be a general phenomenon for these chloro-anions.

With Fe(III), Co(II), Cu(II), Ga(III), UO_2(II) and MoO_4^{2-}, there is considerable lowering of the R_F values on SB-2 paper in comparison with HCl and noticeable but relatively small effects with the cellulose exchangers. Surprisingly there is little difference between HCl and LiCl for ^{7}n(II) and Cd(II), although larger salting-out effects would be expected from previous work with anion exchange resins.

(iv) The adsorption of metal ions from HBr solutions

The papers (washed with 2 *N* HCl and water as usual) were converted to the bromide form by leaving them for 30 min in *N* KBr, washing with distilled water till free of excess bromide and subsequent drying in air. Since oxidation to bromine in the atmosphere is a risk with the bromide form of the papers, they were always freshly prepared and used within 24 h. The solutions of HBr used were prepared freshly each day from colourless batches of conc. HBr (Carlo Erba). The solutions of the metal ions were prepared in 4 *N* HBr from chlorides in the case of the reversibly complexing metal ions. Chlorauric acid was heated on the water bath fors everal hours in 4 *N* HBr to convert it to $HAuBr_4$.

Fig. 4 shows the R_F values on SB-2 paper, DEAE paper, aminoethylcellulose paper and Whatman No. 1 paper for a number of selected ions.

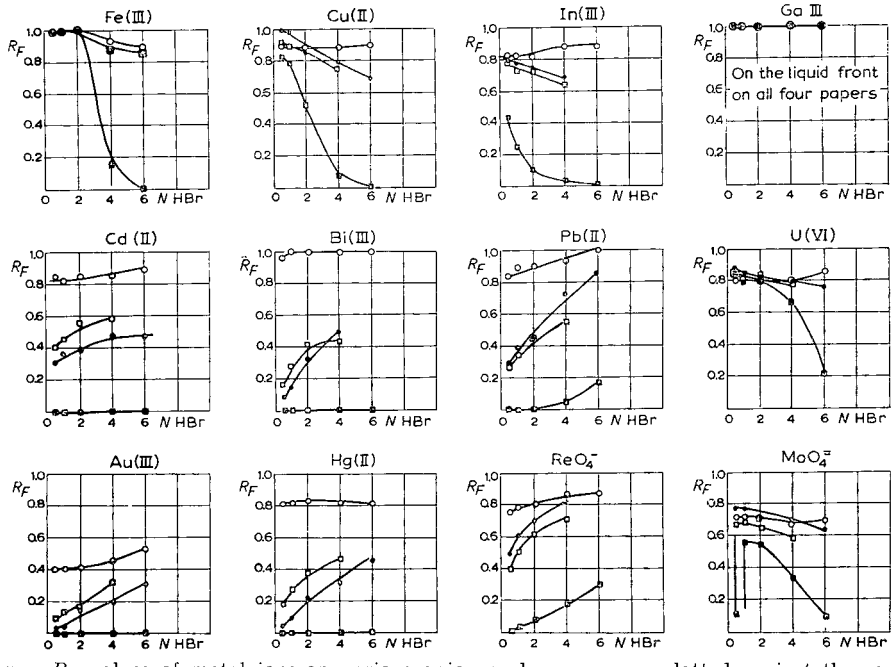


Fig. 4. R_F values of metal ions on various anion exchange papers plotted against the concentration of HBr. Symbols as in Fig. 1.

It is useful to start discussing these results with the perrhenate ion which is neither complexed nor reduced in HBr. Its R_F values are much higher than in the corresponding HCl concentration owing to the stronger affinity of Br^- for the exchanger and the consequent higher displacing action on the ReO_4^- ion. The sequence of the R_F values and the relative values on the various papers indicate that the mechanism of fixation is essentially one of ion exchange with little or no adsorption. Both Pb(II) and In(III) seem to be also retained by ion exchange, while Au(III) (*i.e.* $AuBr_4^-$), Cd(II), Bi(III) and Hg(II) seem to be held by adsorption as well as ion exchange; their R_F values on the DEAE paper being considerably lower than on the aminoethyl-cellulose paper.

The retention of Fe(III) and Cu(II) on the SB-2 paper seems to be due to adsorption since there is hardly any fixation on the cellulose exchangers when the R_F values on the SB-2 are near zero. Gallium is practically unadsorbed as could be expected from solvent extraction and paper chromatography results. Molybdate produces long comets on SB-2 paper at concentrations of HBr below 2 N and its values then decrease slowly. U(VI) is much less adsorbed than from HCl.

CONCLUSIONS

The results given here confirm on the whole the picture of adsorption on anion exchangers presented in the two previous communications, namely that both adsorption and ion exchange contribute to the retention of ions on the various exchangers and that the differences between HCl and LiCl may be interpreted as a "salting-out effect", affecting those ions which are held by adsorption. A qualitative measure of "adsorption" can be found in the differences in R_F values between the DEAE paper and the aminoethyl cellulose paper where the first will "adsorb" more strongly having two additional ethyl groups, while the latter has a higher ion exchange capacity and should retain ions more strongly by ion exchange. The differences between the adsorption on WB-2 (weak base) and SB-2 (strong base) resin papers can be explained by considering the differences in the polymers rather than in their exchange groups. Preliminary work with a cellulose anion exchanger with quaternary ammonium groups (kindly presented by Macherey-Nagel) showed again that the adsorption of $AuCl_4^-$ from 2 N HCl ($R_F = 0.18$) is similar to that on other weak anion cellulose exchangers. It is also very much less than the adsorption on either strong or weak resin paper.

Owing to its simplicity, the method used here for studying ion exchange seems to shed new light on a variety of problems of ion exchange. The results, however, are at best semiquantitative.

Further work is in progress in this laboratory on some of these problems and will be reported here in due course.

SUMMARY

Data on the behaviour of metal ions on various anion exchange papers in HCl, LiCl and HBr are given and the mechanism of the adsorption is discussed.

REFERENCES

[1] M. LEDERER AND L. OSSICINI, *J. Chromatog.*, 13 (1964) 188.
[2] M. LEDERER, *J. Chromatog.*, 13 (1964) 232.
[3] K. A. KRAUS AND F. NELSON, *Proc. Intern. Conf. Peaceful Uses of Atomic Energy, Geneva*, (1955) Vol. VII, p. 113.

CHELATBILDENDE AUSTAUSCHERHARZE

IV. SCHWERMETALLSELEKTIVE KOMPLEXONAUSTAUSCHER AUF POLYAMINPOLYESSIGSÄURE-BASIS

E. BLASIUS UND I. BOCK

Anorganisch-Chemisches Institut der Technischen Universität, Berlin (Deutschland)

(Eingegangen am 26. Juli 1963)

In einer Reihe früherer Veröffentlichungen befassten sich BLASIUS und Mitarbeiter[1-3] mit der Herstellung und Untersuchung der analytischen Eigenschaften von Ionenaustauschern mit selektiven chelatbildenden Gruppen.

Unter den Reagenzien, die infolge Chelatkomplexbildung gewisse Metallionen selektiv binden, spielen die Komplexone, also monomere Polyaminpolyessigsäuren, eine bedeutende Rolle. Die Befähigung zur Ausbildung von Chelaten bleibt erhalten, wenn an einem oder mehreren der N-Atome an Stelle einer Carboxymethylengruppe ein anderer Substituent, etwa eine Alkyl- oder Benzylgruppe[4-8] sitzt. Bei diesen substituierten Komplexonen ist zwar oft eine allgemeine Abnahme der Stabilität, jedoch eine Zunahme der Unterschiede zwischen den Komplexstabilitäten verschiedener Metallionen zu beobachten.

Beide Effekte versprechen ein günstiges Verhalten von Austauschern auf der Basis von Polyaminopolyessigsäuren. Nicht zu hohe absolute Komplexstabilität bedeutet leichtere Entfernung des gebundenen Metallions vom Austauscher, relativ grosse Unterschiede zwischen den Komplexstabilitäten verschiedener Metallionen verbessern den Trenneffekt.

In der Literatur finden sich bereits einige Beispiele für Polymerisationsharze mit komplexonartigen funktionellen Gruppen. Praktische Anwendungen sind jedoch nur von Harzen mit Iminodiessigsäure- und Nitrilotriessigsäure-Gruppen bekannt geworden[9-12]. Bei weiteren erwähnten Komplexonharzen werden keinerlei nähere Angaben über Herstellung, Qualität und Anwendung gemacht[13].

Im folgenden wird die Herstellung von Polyaminpolyessigsäure-Austauscherharzen, die Untersuchung ihrer komplexbildenden Eigenschaften und ihre analytische Anwendung beschrieben.

Die Herstellung der Austauscher erfolgte stufenweise: chlormethyliertes, mit Divinylbenzol vernetztes Polystyrol wurde mit einem Polyamin umgesetzt und das als Zwischenprodukt entstandene Aminharz durch Reaktion mit Chloressigsäure carboxymethyliert.

Es wurden verschiedene homologe Polyamine, nämlich Äthylendiamin, Diäthylentriamin und Triäthylentetramin als Grundform der Austauschergruppe eingesetzt und mehrere unterschiedlich stark vernetzte Gerüstpolymerisate verarbeitet (2%, 4% und 8% Divinylbenzolgehalt).

DARSTELLUNG DER AUSTAUSCHER

Auf Grund der Ergebnisse zahlreicher Vorversuche wurde folgende optimale Darstellungsmethode entwickelt:

20 g lufttrockenes chlormethyliertes Polymerisat werden in 90 ml Dioxan auf dem Wasserbad 30 min unter Rückflusskühlung erhitzt. Das überstehende Dioxan wird dekantiert.

Das gequollene, dioxanfeuchte Harz wird mit 100 ml des gewünschten Amins versetzt und in einem Jodzahlkolben kurze Zeit bis zur beginnenden Erwärmung geschüttelt. Um einen weiteren Temperaturanstieg auszuschliessen, wird das Reaktionsgemisch 15 Stunden im Eisschrank bei 4° aufbewahrt, dann bei Raumtemperatur in einer Schüttelmaschine 10 Tage lang bewegt.

Das aminierte Harz wird neutral gewaschen und durch Behandlung mit 2 *N* H_2SO_4 in die Sulfatform überführt. Zwecks Wiederherstellung der Basenform wird das Harz mit 1 *N* NaOH behandelt, bis keine Sulfationen mehr nachzuweisen sind. Nach gründlichem Auswaschen wird das Präparat bei Raumtemperatur an der Luft getrocknet (ca. 10 Tage).

20 g des so erhaltenen Aminharzes werden in 250 ml Dioxan durch einstündiges Erhitzen unter Rückflusskühlung auf dem Wasserbad vorgequollen und anschliessend über Nacht stehen gelassen. Das Dioxan wird weitgehend abgegossen und zu dem Harz ein unter Eiskühlung hergestellter Brei aus 285 g (3 Mol) Monochloressigsäure und 160 g (1.5 Mol) Na_2CO_3 in 100 ml Wasser gegeben. Für alle drei Aminharze wird die gleiche Menge Chloressigsäure eingesetzt, da sie in jedem Fall einen reichlichen Überschuss darstellt. Die Mischung wird 2 Tage bei Zimmertemperatur gerührt, dann auf dem Wasserbad langsam auf 95° gebracht. Der pH-Wert der Lösung, der ursprünglich etwa 7.5 beträgt, sinkt anfangs rasch auf 3–4, so dass mehrere Zugaben von Natriumcarbonat (in Abständen von 0.5–1 Stunde) erforderlich sind, um die Reaktion bei pH 7–8 durchzuführen. Nach der letzten Sodazugabe wird die Mischung noch 6 Stunden lang bei 95° gerührt, ohne dass der pH-Wert weiter abnimmt.

Der Austauscher bleibt über Nacht unter der Reaktionsflüssigkeit stehen, wird dann mit Wasser alkalifrei gewaschen und schliesslich zwecks Überführung in die H^+-Form mit 2 *N* H_2SO_4 behandelt. Das gründlich gewaschene, säurefreie Endprodukt wird 10 Tage lang bei Raumtemperatur an der Luft getrocknet.

Die Austauscher zeichnen sich durch grosse Beständigkeit aus. Auch nach langem Gebrauch ist keine Änderung des Stickstoffgehalts und kein Kapazitätsverlust festzustellen. Die Anwendung der Austauscherharze in Säulen wird nicht durch die bei den meisten anderen Chelataustauschern übliche grosse Volumenänderung bei Umladung beeinträchtigt.

BESTIMMUNG DER AUSTAUSCHEREIGENSCHAFTEN

Bei der Reaktion eines chlormethylierten, vernetzten Styrol-Polymerisats mit Polyäthylenpolyaminen enstehen Produkte, bei denen das Aminmolekül teils über eine, teils über mehrere Methylenbrücken an das Harzgerüst gebunden ist, wie sich aus nach der Kjeldahl-Methode erhaltenen Stickstoffwerten folgern lässt. Die Anzahl der Verbindungsstellen mit dem Gerüst ist bei den einzelnen Aminen verschieden. Der Vernetzungsgrad hat nur geringen Einfluss auf den Einbau der Aminreste. Erst

bei 8 %iger Vernetzung machen sich für Triäthylentetramin sterische Behinderungen bemerkbar.

Bei der Carboxymethylierung der aminierten Zwischenprodukte wird nur ein Teil der zur Verfügung stehenden Amin-Wasserstoffatome substituiert. Auch diese Erscheinung hängt wenig vom Vernetzungsgrad, mehr vom vorliegenden Amin ab.

Zur Charakterisierung der Präparate hinsichtlich ihrer Austausch-, insbesondere Komplexbildungsfähigkeit, wurden Neutralisationstitrationen sowohl ohne, als auch mit Zusatz komplexierbarer Kationen durchgeführt, sowie die Beladung mit verschiedenen Metallionen in Abhängigkeit vom pH-Wert verfolgt. Als Modellkationen dienten Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} sowie Mg^{2+}. Die Aufnahme der Neutralisations- und auch der Beladungskurven erfolgte im batch-Verfahren.

(a) Neutralisationskurven

Die hergestellten Austauscher verhalten sich bei der Titration wie schwache mehrbasige Säuren, wie aus Lage und Form der Neutralisationskurven ohne Zusatz von Kationen höherer Ionenladung hervorgeht (siehe Fig. 1–3).

Die Kurven beginnen bei etwa pH 4 und weisen zwei bzw. drei Puffergebiete und Sprünge auf, die infolge Überlagerungserscheinungen auf Grund des uneinheitlichen Baus der Festgruppen mehr oder weniger schlecht ausgebildet sind. Zwischen den verschiedenen Amintypen sind Abstufungen in der Acidität zu erkennen.

Zur Komplexbildung befähigte Metallionen höherer Ionenladung können an die carboxymethylierten Amin-Gruppen über mehrere Chelatringe gebunden werden. Solche Kationen werden daher stark bevorzugt, so dass der Protonenaustritt bei niedrigeren pH-Werten erfolgt.

Die Fig. 1–3 zeigen neben den einfachen Neutralisationskurven der Austauscher die Kurven, die sich bei der Neutralisation in Gegenwart verschiedener Me^{2+}-Ionen ergeben.

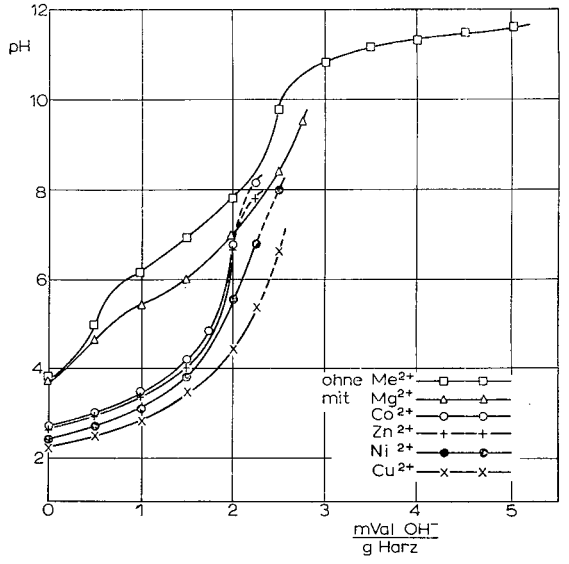


Fig. 1. Neutralisation von Äthylendiaminpolyessigsäure-Austauscher.

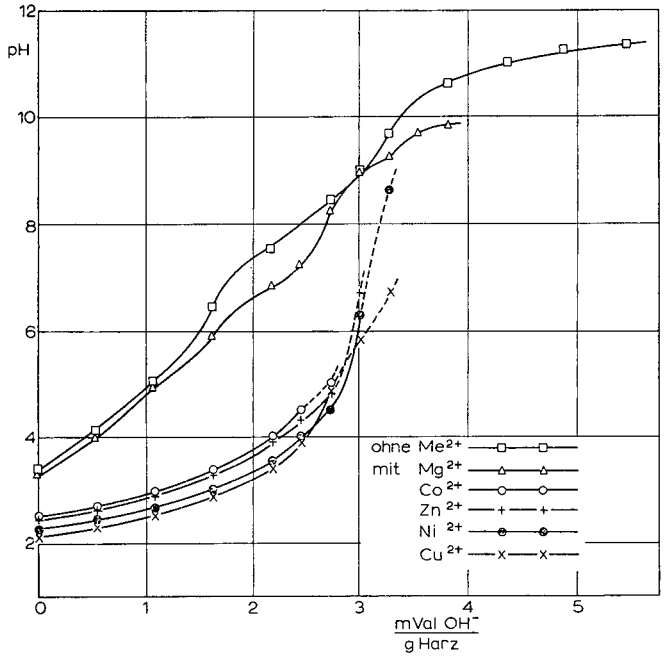


Fig. 2. Neutralisation von Diäthylentriaminpolyessigsäure-Austauscher.

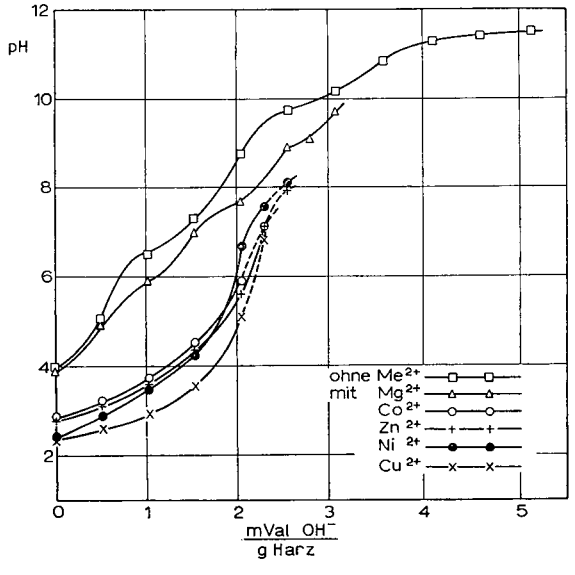


Fig. 3. Neutralisation von Triäthylentetraminpolyessigsäure-Austauscher.

Fasst man die Austauscher als zweiprotonige (Triäthylentetraminpolyessigsäure-Austauscher als dreiprotonige) Festsäuren auf, so werden durch die Komplexbildung mit Schwermetallionen beide (bzw. die ersten beiden) Protonen betroffen. Beide dissoziieren in Gegenwart von Me^{2+}-Ionen etwa gleichzeitig. Die Kurvenverschiebung ist ein Mass für die relative Stabilität der gebildeten Komplexe und zeigt, dass zwischen Erdalkali- und Schwermetallionen-Komplexen beträchtliche, jedoch nur unbedeutende Unterschiede innerhalb der Gruppe der Übergangselemente selbst bestehen. Die Reihenfolge der Komplexstabilität ($Co^{2+} < Zn^{2+} < Ni^{2+} < Cu^{2+}$) ist bei allen drei Austauschern gleich. Die unterschiedliche Verschiebung bei den einzelnen Austauschertypen entspricht der Abhängigkeit der Komplexstabilität von der Zahl der zur Verfügung stehenden Ligandstellen bzw. der möglichen Chelatringe.

Für jede Neutralisationskurve ohne Zusatz von Me^{2+}-Ionen wurden mindestens elf Einzelansätze gemacht, wobei jeweils 0.5 g lufttrockener Austauscher in H^+-Form in einem gut verschliessbaren Erlenmeyerkolben mit berechneter Menge Wasser, 1 *N* NaCl-Lösung und, als Titrationsflüssigkeit, 0.1 *N* NaOH in steigenden Mengen von 0 bis 20 ml versetzt wurde. Die zugegebenen Mengen NaCl-Lösung und Wasser waren entsprechend dem NaOH-Anteil des Ansatzes so berechnet, dass die Gesamtkonzentration des Ansatzes an Na^+ 0.5 *M* bei einem Lösungsvolumen von 100 ml für 0.5 g Austauscher betrug. Die Ansätze wurden unter Schütteln 7 Tage aufbewahrt, dann die pH-Werte der Lösungen potentiometrisch bestimmt. Diese pH-Werte, aufgetragen gegen die pro Gewichtseinheit getrockneten Austauschers zugesetzten Äquivalente Lauge, liefern die Titrationskurven.

Für die Aufnahme von Neutralisationskurven in Gegenwart komplexierbarer Me^{2+}-Ionen wurde wie oben angegeben verfahren, wobei jedoch je Probe 1 mVal Me^{2+}-Ion zugesetzt wurde. Die Gesamtkonzentration an Na^+ und Me^{2+} wurde jeweils auf 0.5 *N* eingestellt. Um eine Ausfällung der zugesetzten Kationen möglichst zu verhindern, erfolgte der Laugezusatz erst nach mehrstündigem Stehen der Ansätze, bei grösseren Laugemengen im allgemeinen portionsweise. Die Höchstmenge Lauge, die zugesetzt werden konnte, ohne dass eine bleibende Hydroxidfällung auftrat, richtete sich nach dem anwesenden Me^{2+}-Ion. Da die Reaktion der zugesetzten Me^{2+}-Ionen mit den Austauschergruppen wesentlich langsamer abläuft als die mit OH^--Ionen, können trotzdem die letzten Messpunkte der unter Me^{2+}-Zusatz aufgenommenen Kurven bereits in geringem Masse durch Hydroxidfällung beeinflusst sein. Die betroffenen Kurvenabschnitte sind schraffiert gezeichnet.

(*b*) *Beladungskurven*

Für die Bindung der komplexbildenden Kationen, gemessen bei zwei verschiedenen Me^{2+}-Konzentrationen (im Verhältnis 1:2), ergibt sich bei allen drei Austauschern im wesentlichen das gleiche Bild. In Übereinstimmung mit den Ergebnissen der Neutralisationstitrationen zeigen alle eine besonders hohe Selektivität für Cu^{2+}, hinter der die Bindung der Gruppe Co^{2+}, Zn^{2+}, Ni^{2+} überall in ähnlichem Umfang zurückbleibt. Die Austauschwerte innerhalb dieser Gruppe unterscheiden sich nur wenig. Die Mg^{2+}-Aufnahme weicht erheblich von der Bindung der Ionen der Übergangselemente ab, am stärksten bei Diäthylentriaminpolyessigsäure-Austauscher.

Die aufgenommene Me^{2+}-Menge hängt bei niedrigen pH-Werten kaum von der Me^{2+}-Konzentration in der Lösung ab, sondern wird in erster Linie durch die Stärke der Komplexbildung mit den Austauschgruppen bestimmt.

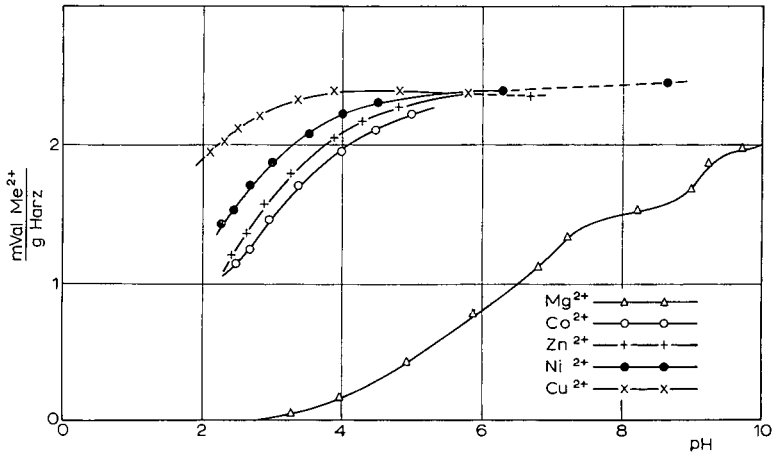


Fig. 4. Beladung von Diäthylentriaminpolyessigsäure-Austauscher bei Einsatz von 1 mVal Me^{2+}.

Von den untersuchten Austauschern zeichnet sich Diäthylentriaminpolyessigsäure-Austauscher als stärkster Chelatbildner aus. Die Komplexwirksamkeit des Triäthylentetraminpolyessigsäure-Austauschers ist nur wenig grösser als diejenige des Äthylendiaminpolyessigsäure-Austauschers.

Fig. 4. zeigt die an Diäthylentriaminpolyessigsäure-Austauscher gemessene Beladung bei Einsatz von 1 mVal Me^{2+}.

Die Schwermetallionen werden bereits bei niedrigen pH-Werten (unterhalb pH 2.5) in beträchtlichen Mengen vom Austauscher gebunden. Nach anfänglich steilem Anstieg der Beladungskurven werden je nach Stärke der gebildeten Komplexe im Bereich von ca. pH 3.5–5.5 die Endwerte der Me^{2+}-Aufnahme erreicht, die entsprechend der geringen zugesetzten Mengen Me^{2+} unter der totalen Austauschkapazität liegen.

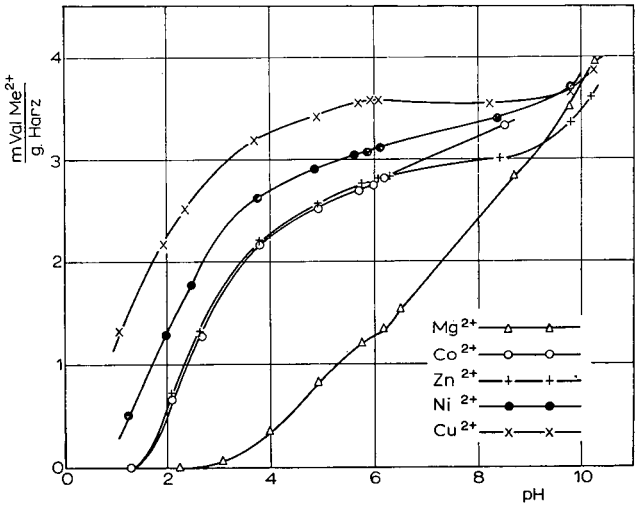


Fig. 5. Beladung von Diäthylentriaminpolyessigsäure-Austauscher bei Einsatz von 2 mVal Me^{2+}.

Die Mg^{2+}-Aufnahme beginnt dagegen erst bei pH 3.5. Der Anstieg der Beladungskurven verläuft unter Andeutung von Sprüngen weniger steil und erstreckt sich über ein viel grösseres pH-Gebiet, bis auch hier der Endwert erreicht wird.

Bei Einsatz der doppelten Menge Me^{2+} ergibt sich das in Fig. 5 dargestellte Diagramm.

Auch hier ist für Schwermetallionen ein steiler Beginn der Me^{2+}-Aufnahme zu erkennen, der, abhängig von der Komplexstabilität, in ein mehr oder weniger saures Gebiet fällt.

Die Anfänge der Kurven decken sich ungefähr mit den Anfängen der Beladungskurven bei Einsatz von nur 1 mVal Me^{2+}. In dem Masse, wie die Dissoziation der Komplexe nachlässt, also erst bei höheren pH-Werten, wirkt sich die grössere Me^{2+}-Konzentration in einer erhöhten Me^{2+} Aufnahme aus. Die Zunahme ist umso grösser, je stabiler der Komplex ist.

Zur Aufnahme der Beladungskurven wurden die Austauscher mit den entsprechenden Kationen in gepufferter Lösung von pH 1–11, die in der Lage war, die Kationen weitgehend komplex in Lösung zu halten, ins Gleichgewicht gesetzt.

Es wurden 0.25 *M* Ammonacetat–Salzsäure- bzw. 0.25 *M* Ammonacetat–Ammoniak-Puffer verwendet, welche bis zu pH 8–9 alle betrachteten Metallionen einwandfrei in Lösung hielten. Die Puffer wurden durch Mischen wechselnder Mengen 0.25 *M* Ammonacetatlösung und — je nach gewünschtem pH-Wert — 0.25 *M* HCl bzw. 0.25 *M* NH_3 hergestellt.

Da für die Beladungskurven ohnehin die pH-Werte der Gleichgewichtslösungen benutzt werden, wurde darauf verzichtet, die Austauscher vor der Me^{2+}-Zugabe mit den Puffern ins Gleichgewicht zu bringen.

Für jeden Ansatz wurden 0.5 g lufttrockener Austauscher in H^+-Form in einem gut verschliessbaren Erlenmeyerkolben mit 10 ml einer 0.1 bzw. 0.2 *N* Lösung des jeweiligen Me^{2+}-Salzes und 100 ml Puffer von entsprechendem pH-Wert versetzt. Nach 7-tägigem Aufbewahren unter Schütteln wurde der pH-Wert der Lösung gemessen und der Gehalt an nicht ausgetauschtem Me^{2+} durch komplexometrische Titration eines aliquoten Teils der überstehenden Lösung bestimmt. Die Differenzen zwischen diesen Werten und der eingesetzten Menge Me^{2+}, umgerechnet auf 1 g entwässerten Austauschers, sind in den Beladungskurven als aufgenommene Menge Me^{2+} gegen die pH-Werte der Gleichgewichtslösungen aufgetragen.

TRENNUNGEN

Die pH-abhängige Selektivität der Austauscher lässt sich vorteilhaft zu Kationentrennungen ausnutzen. Durch besonders geringen Material- und Arbeitsaufwand zeichnet sich jene Methode aus, die Kationenmischung bei einem solchen pH-Wert über die Austauscherpackung zu geben, bei dem das eine Ion quantitativ gehalten, das andere dagegen nicht oder höchstens geringfügig gebunden wird. Dadurch ist es möglich, mit wesentlich weniger Austauschersubstanz und damit auch kleineren Mengen an Wasch- und Elutionsmitteln auszukommen, als für eine chromatographische Trennung erforderlich sind, wenn beide Ionen am Austauscher haften.

Nach diesem Prinzip wurden zahlreiche Zweistoff-Trennungen durchgeführt, z.B. Cu^{2+}–Co^{2+}, Cu^{2+}–Zn^{2+}, Cu^{2+}–Mg^{2+}. Das molare Mischungsverhältnis war im allgemeinen 1:1, wurde aber bei der Trennung Cu^{2+}–Mg^{2+} von 20:1 bis 1:1000 variiert.

Der für die jeweilige Trennung in Frage kommende pH-Wert richtet sich nach den anwesenden Kationen und dem verwendeten Austauscher und wird aus den Beladungskurven abgeleitet. Um möglichst genau den optimalen pH-Wert einzuhalten, erfolgen Aufgabe der Me^{2+}-Mischung und Auswaschen des nicht gebundenen Metallions zweckmässigerweise mit Pufferlösungen. Bei den verwendeten Pufferlösungen handelt es sich um die gleichen Ammonacetat–Salzsäure-Puffer, die auch bei der Aufnahme der Beladungskurven benutzt werden.

Der Verlauf der Trennungen wird in Form von Elutionskurven dargestellt, in denen die Me^{2+}-Konzentration im Eluat gegen die Menge Elutionsmittel, ausgedrückt durch den Quotienten aus Eluatvolumen V_E und Säulenbettvolumen V_B, aufgetragen ist.

Vor Aufgabe des Me^{2+}-Gemisches werden die Austauschersäulen mit Wasser oder Puffer von einem für den jeweiligen Versuch in Aussicht genommenen pH-Wert solange gespült, bis der Ablauf denselben pH-Wert wie die aufgegebene Flüssigkeit hat.

Nach Ablauf des Puffers bis zum oberen Rand der Packung wird die zu trennende Zweistoff-Mischung in den der Säule aufsitzenden Trichter eingefüllt. Anschliessend wird die Packung mit dem vorgesehenen Waschmittel kontinuierlich gewaschen.

Für den in Fig. 6 dargestellten Modellversuch wurden Fraktionen zu je 100 ml bzw. 250 ml gesammelt und jede Fraktion unter den erforderlichen Bedingungen einzeln auf jedes Me^{2+} der Trennmischung komplexometrisch titriert[14], was den Nachweis der An- bzw. Abwesenheit des zweiten Me^{2+} in der Waschfraktion erbrachte. Diese Art des Nachweises erwies sich als weit empfindlicher als eine Prüfung mit den in der qualitativen Analyse gebräuchlichen Reagenzien.

Nach restloser Entfernung des ersten Metallions mit Waschlösung wurden noch ein oder zwei weitere Fraktionen Waschmittel aufgefangen, welche auf das zweite, eventuell bereits durchgebrochene Me^{2+} geprüft wurden.

Danach wird das zweite Me^{2+} mit der vorgesehenen Säure abgelöst und im Eluat komplexometrisch bestimmt. In jeder ersten Eluatfraktion sollte vorsichtshalber auf Rückstände des mit der Waschlösung abgetrennten Me^{2+} geprüft werden.

Nach Beendigung der Elution werden die Säulen mit Wasser gewaschen, bis der pH-Wert im Ablauf zwischen 3.5 und 4 beträgt. Danach sind die Austauscher ohne Regeneration wieder einsatzfähig.

Ein interessantes Beispiel für die Anwendung der Austauscher sei an einer Serie von Trennungen des Systems Cu^{2+}–Mg^{2+} bei extremen Konzentrationsverhältnissen an einem Diäthylentriaminpolyessigsäure-Austauscher gezeigt. Das molare Mischungsverhältnis wird von 20:1 bis 1:1000 variiert (Fig. 6, Tabelle I).

Die Versuche zeigen, dass es mit Hilfe der beschriebenen Polyaminpolyessigsäure-Austauscher auf einfache Art möglich ist, Spuren von Schwermetallionen aus Erdalkalilösungen zu entfernen. Der Spurenbestandteil, hier Cu^{2+}, wird auch bei extremer Verdünnung stets sauber aus der Mischung abgetrennt. Mg^{2+} wird auch bei Übergabe sehr grosser Mengen nicht vom Austauscher gebunden. Es erscheint gleich im Filtrat, so dass also keine fraktionierte Elution erforderlich ist. Zwischen das Mg^{2+}-haltige Filtrat und das Cu^{2+}-haltige Eluat können beliebig viele Waschfraktionen eingeschaltet werden, in denen keins der beiden Ionen nachzuweisen ist.

Nach dem angeführten Prinzip können auch die Ionen Ni^{2+}, Zn^{2+} oder Co^{2+} von Cu^{2+} abgetrennt werden. Die Versuchsbedingungen sind entsprechend zu variieren.

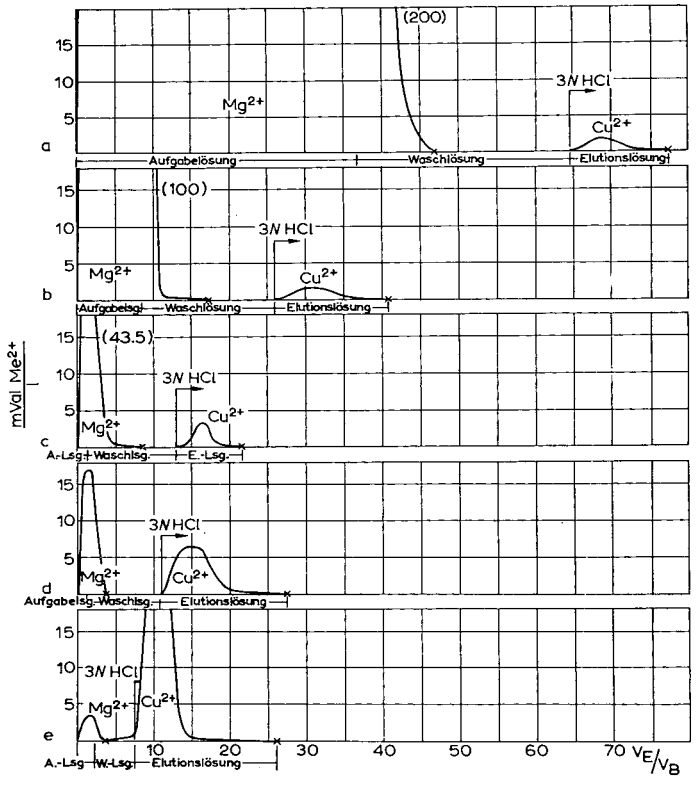


Fig. 6. Trennung von Cu^{2+}–Mg^{2+} über Diäthylentriaminpolyessigsäure-Austauscher.

TABELLE I

VERSUCHSDATEN ZU FIG. 6

	(a)	*(b)*	*(c)*	*(d)*	*(e)*
Harzhöhe in der Säule (H_B) (cm)	23.5	20	20	23.5	23.5
Querschnitt der Säule (E_B) (cm^2)	1.15	1.15	1.15	1.15	1.15
Harzvolumen (V_B) (ml)	27	23	23	27	27
Austauschermenge (g)	18	15	15	18	18
Mischungsverhältnis Cu^{2+}: Mg^{2+}	1:1000	1:100	1:10	1:1	20:1
Aufgabe (mVal) an					
Cu^{2+}	0.2	0.2	0.2	1	4
Mg^{2+}	200	20	2	1	0.2
Konzentration der Aufgabelösung (M) an					
Cu^{2+}	0.0001	0.0005	0.0032	0.0167	0.033
Mg^{2+}	0.1	0.05	0.032	0.0167	0.00165
pH der Waschlösung	1.4	1.4	1.4	1.5	1.3
Elutionsmittel	3 *N* HCl	3 *N* HCl	3 *N* HCl	3 *N* HCl	3 *N* HCl
Durchflussgeschwindigkeit (ml/min)	1.2	0.8	1.0	0.5	0.1

DANK

Wir danken der Verwaltungsstelle für ERP-Vermögen und der Deutschen Forschungsgemeinschaft für die Bereitstellung von Mitteln und Apparaturen, sowie Herrn Dr. W. BUSSE von der Permutit AG, Berlin, für die Überlassung von chlormethylierten Styrolperlpolymerisaten.

ZUSAMMENFASSUNG

Durch Umsetzung von vernetzten, chlormethylierten Styrolpolymerisaten mit Polyäthylenpolyaminen und darauffolgende Carboxymethylierung entstehen Ionenaustauscher mit chelatbildenden Festgruppen.

Da die Polyäthylenpolyamine bei der Aminierungsreaktion je nach Grösse teils einfach, teils mehrfach an das Harzgerüst gebunden werden und die Carboxymethylierung unvollständig ist, sind die einzelnen Festgruppen eines Austauschers nicht einheitlich gebaut. Daher ist die Komplexbildung am Austauscher nicht eindeutig zu beschreiben.

An Hand der Neutralisations- und Beladungskurven sind jedoch deutliche Abstufungen in der Acidität und Komplexwirksamkeit der durch Einbau verschiedener Amine hergestellten Harztypen zu erkennen.

Die Austauscher lassen sich vorteilhaft mit geringem Material- und Arbeitsaufwand zur Trennung von Metallionen, besonders auch bei extremen Konzentrationsverhältnissen, verwenden, wie in zahlreichen Zweistofftrennungen verschiedener Me^{2+}-Kombinationen im Säulenverfahren nachgewiesen wurde, indem die Kationenmischung bei einem solchen pH-Wert über die Austauscherpackung gegeben wird, bei dem das eine Ion quantitativ gehalten, das andere nicht oder höchstens geringfügig gebunden wird.

SUMMARY

Ion exchangers containing chelating functional groups are obtained when crosslinked chloromethylated styrene polymers are treated with polyethylenepolyamines and subsequently carboxymethylated with chloroacetic acid.

According to their length the polyethylenepolyamines are linked to the matrix by one or more bridges by the amination reaction. The carboxymethylation of the final product is incomplete. Therefore the chelating groups of a given exchanger do not have the same structure and it is impossible to give an exact description of the complex formation of the resins.

Nevertheless neutralization and adsorption curves of the resins show differentiations in the acidity and complexing ability of the three resin types containing different amines.

In a time- and material-saving procedure the ion exchangers can be used to separate metal ions present in extreme concentration ratios. A column procedure was developed by which a number of two-element separations was performed. The cation mixture was passed through the resin bed at a pH value at which one ion is adsorbed quantitatively by the resin, while the second ion is not or only slightly bound.

LITERATUR

[1] E. BLASIUS UND G. OLBRICH, *Z. Anal. Chem* , 151 (1956) 81.
[2] E. BLASIUS UND B. BROZIO, *Z. Anal. Chem.*, 192 (1963) 364.
[3] E. BLASIUS UND M. LASER, *J. Chromatog.*, 11 (1963) 84.
[4] A. E. FROST, JR. UND A. E. MARTELL, *J. Am. Chem. Soc.*, 72 (1950) 3743.
[5] S. CHABEREK, JR. UND A. E. MARTELL, *J. Am. Chem. Soc.*, 77 (1955) 1477.
[6] F. H. SPEDDING, J. E. POWELL UND E. J. WHEELWRIGHT, *J. Am. Chem. Soc.*, 78 (1956) 34.
[7] Refined Products Corp., *Brit. Pat.*, 767,162 (1957).
[8] F. C. BERSWORTH, *U.S. Pat.*, 2,630,455 (1953).
[9] R. A. MOCK UND L. R. MORRIS, *U.S. Pat.*, 2,840,603 (1958).
[10] L. R. MORRIS, R. A. MOCK, C. A. MARSHALL UND I. H. HOWE, *J. Am. Chem. Soc.*, 81 (1959) 377.
[11] R. HERING, *Z. Chem.*, 3 (1963) 30, 69, 108, 153.
[12] R. HERING, *J. Prakt. Chem.*, 14 (1961) 285.
[13] L. WOLF UND R. HERING, *Chem. Tech. (Berlin)*, 10 (1958) 661.
[14] G. SCHWARZENBACH, Die Komplexomctrische Titration, *Die Chemische Analyse*, Bd. 45, Ferdinand Enke-Verlag, Stuttgart, 1960.

ION EXCHANGE PROCEDURES

II. SEPARATION OF ZIRCONIUM, NEPTUNIUM AND NIOBIUM*,**

JAMES H. HOLLOWAY*** AND FREDERICK NELSON

Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. (U.S.A.)

(Received May 18th, 1963)

This procedure was devised for separation and individual isolation of trace amounts of neptunium and niobium from macro-amounts of zirconium. It makes use of the fact that Zr(IV) in HCl–HF media at high HCl concentrations is essentially non-adsorbable[2] by anion exchange resins under conditions where Np(VI) and Nb(V) are strongly adsorbed; these may then be sequentially eluted.

TYPICAL SEPARATION

Separation of milligram amounts of Zr(IV) and trace amounts of Np(VI) and Nb(V) is illustrated in Fig. 1. A 0.38 *M* $ZrOCl_2$ (*ca.* 34 mg Zr/ml) solution was prepared by dissolving $ZrOCl_2 \cdot 8\ H_2O$ in warm 6 *M* HCl–1 *M* HF. When dissolution was complete, tracer ^{95}Zr–^{95}Nb and ^{238}Np were added. Chlorine gas was bubbled through the solution for a few minutes to assure oxidation of neptunium to Np(VI).

A 0.67 $cm^2 \times 3$ cm resin column (bed volume 2 ml) was prepared with Dowex 1-X10, —400 mesh resin. The resin as a slurry in water was treated with chlorine gas before preparing the column to insure strongly oxidizing conditions in the bed. The column was pretreated with 5 ml of 6 *M* HCl–1 *M* HF–Cl_2 which was also used for elution after addition of the Zr–Np–Nb sample (1 ml). Flow rate was 0.8 cm/min. Effluent fractions (0.5 ml) were collected and counted. Zirconium was non-adsorbed and appeared in maximum concentration at less than 1 column volume (c.v.) of effluent while Np(VI) and Nb(V) remained essentially completely adsorbed. A small amount ($< 2\%$) of Np appeared in the Zr-fraction. The neptunium remaining on the column was selectively eluted with 0.5 *M* HCl–1 *M* HF–Cl_2 solution. Niobium was removed with 4 *M* HNO_3–1 *M* HCl–0.2 *M* HF.

DISCUSSION

This separation requires neptunium to be essentially completely oxidized to Np(VI). Since Np(V) is not adsorbed from HCl solutions[3], and Np(IV) is weakly adsorbed[4] from 6 *M* HCl–HF mixtures, the appearance of a small amount of neptunium in the zirconium fractions indicates that some reduction may occur even in the presence of

* This document is based on work performed at the Oak Ridge National Laboratory, Oak Ridge, Tenn., operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

** For Part I see ref. 1.

*** United States Air Force.

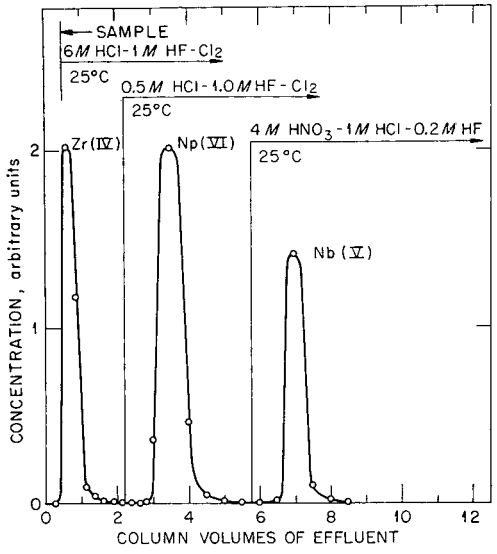


Fig. 1. Separation of milligram amounts of Zr(IV) and trace amounts of Np(VI) and Nb(V) (Dowex 1-X10, —400 mesh, 0.67 cm^2 × 3 cm column).

chlorine. Under the conditions described, however, neptunium leakthrough is relatively small, *i.e.*, less than 2%.

A fine mesh resin (—400) is used to permit separation with a shallow bed at reasonably rapid flow rates; with the resin used in this procedure, sharp elution bands are observed.

PROCEDURE

(*a*) *Materials and reagents*

Resin. Dowex 1-X10 (—400 mesh), chloride form.

Apparatus. A section of plastic tubing 0.9 cm I.D. and 12 cm in length is used to prepare the column. The tubing is pulled out to a tip at one end and a porous Teflon plug inserted to retain the resin. Additional apparatus used are plastic test tubes, Teflon evaporating dishes, and plastic transfer pipettes.

Column. Resin bed: 0.67 cm^2 × 3.0 cm. Flow rate: *ca.* 0.8 cm/min. Temperature: 25°. Effluent volumes (column volumes = c.v.):

Zr fraction: 3 c.v. (6 ml) of solution I.

Np fraction: 3 c.v. (6 ml) of solution II.

Nb fraction: 3 c.v. (6 ml) of solution III.

Solutions. (I): 6 *M* HCl–1 *M* HF–Cl_2; (II): 0.5 *M* HCl–1 *M* HF; (III): 4 *M* HNO_3–1 *M* HCl–0.2 *M* HF.

(*b*) *Sample preparation*

The sample containing milligram amounts of zirconium is dissolved by standard methods, evaporated to near dryness, and residue taken up in about 1 ml of 6 *M* HCl–1 *M* HF–Cl_2. If solids are present, the solution is warmed to hasten their dissolution. The sample is transferred to a plastic tube and a small stream of chlorine gas ubbled through it for *ca.* 3 min.

(c) Column operation

Resin as a slurry in water is chlorinated about 3 min. with Cl_2 gas and then added to the plastic column until a resin bed about 3 cm in length (*ca.* 2.0 ml) is formed. The column is pretreated with at least 2 c.v. (4 ml) of 6 *M* HCl–1 *M* HF–Cl_2 solution and the sample added. Flow rate is controlled by air pressure to about 0.8 cm/min. After the sample has passed into the resin bed, 0.5 c.v. of 6 *M* HCl–1 *M* HF–Cl_2 are added as wash, taking care not to disturb the resin at the top of the bed. When the wash solution has passed into the bed, an additional 3 c.v. (4 ml) of eluent are added and elution continued. The effluent is collected in a plastic test tube and contains > 99% of the Zr(IV) while Np(VI) and Nb(V) remained adsorbed.

Np(VI) is eluted by passing 3 c.v. (6 ml) of 0.5 *M* HCl–1 *M* HF through the resin bed. The effluent is collected in a plastic tube and contains > 98% of the Np. Nb(V) is removed with 3 c.v. (6 ml) of 4 *M* HNO_3–1 *M* HCl–0.2 *M* HF. The column may be regenerated by treating with 6 ml of 6 *M* HCl–1 *M* HF–Cl_2.

Column operation time for the separation of Zr(IV) and Np(VI) into individual fractions is about 20 min; an additional 10 min is required to remove Nb(V).

ACKNOWLEDGEMENT

The authors are indebted to Dr. K. A. KRAUS for valuable comments and discussion.

SUMMARY

An anion exchange procedure for separating trace amounts of neptunium and niobium from each other and from macro-amounts of zirconium is described.

REFERENCES

1 F. NELSON, T. MURASE AND K. A. KRAUS, *J. Chromatog.*, 13 (1964) 503.
2 K. A. KRAUS AND G. E. MOORE, *J. Am. Chem. Soc.*, 73 (1951) 9.
3 Y. MARCUS, F. NELSON, H. O. PHILLIPS AND K. A. KRAUS, *Oak Ridge Natl. Lab. Rept.*, ORNL 2584 (1958).
4 L. WISH, *Anal. Chem.*, 31 (1959) 326.

ION EXCHANGE PROCEDURES

III. SEPARATION OF URANIUM, NEPTUNIUM AND PLUTONIUM*,**

FREDERICK NELSON, DONNA C. MICHELSON AND JAMES H. HOLLOWAY***

Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. (U.S.A.)

(Received May 18th, 1963)

Numerous methods have been investigated in recent years for separating uranium and the trans-uranium elements. For a recent review of analytical methods see HOFFMAN[2]. The present procedure, while based on existing methods[3,4], appears preferable because it is rapid, simple and permits quantitative separation of the elements. The method utilizes anion exchange in HCl for separating U, Np and Pu from "non-adsorbable" elements which include alkali metals, alkaline earths, rare earths, trivalent actinides and a number of other elements such as Al, Sc, Y, Ac, Th and Ni.

Adsorption of the uranides by anion exchangers from HCl solutions can occur in either the +4 or +6 oxidation states but not in the +3 or +5 states[5]. Adsorption and sequential elution thus can be achieved through control of the oxidation states. In this procedure, uranium is adsorbed in the +6 state while, with the mild oxidizing conditions used in the sample treatment, neptunium and plutonium are adsorbed presumably in the +4 state. Plutonium is selectively reduced to Pu(III) and eluted under conditions where Np(IV) and U(VI) remain adsorbed. The latter are then eluted in separate fractions with HCl–HF mixtures.

In a previous procedure[1], neptunium was adsorbed as Np(VI) from HCl–HF mixtures with chlorine as oxidizing agent. Adsorption of neptunium as Np(IV) from HCl solutions is often preferable because Np(VI) is difficult to maintain in either HCl or HCl–HF solutions and some leak-through may occur through reduction. While removal of neptunium as Np(IV) could be carried out with 4 *M* HCl, the present procedure employs HCl–HF mixtures because they give sharper bands and more rapid removal. In this respect Np(IV) is similar to U(IV), as expected[6].

TYPICAL SEPARATION

A typical separation is illustrated in Fig. 1. A mixture of ^{237}U, ^{238}Np, $^{238-239}Pu$ tracers and some non-adsorbable fission products in 9 *M* HCl–0.05 *M* HNO_3 was prepared. The solution was warmed to insure oxidation of the heavy elements and added to a 0.25 cm^2 × 2 cm column of Dowex 1-X10, —400 mesh, pretreated with 9 *M* HCl. The column was operated at 50° to improve exchange rates. After the sample had drained into the resin bed, elution was carried out with 5 column volumes (c.v.) of 9 *M* HCl

* This document is based on work performed at the Oak Ridge National Laboratory, Oak Ridge, Tenn., operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

** For Part II see ref. 1.

*** United States Air Force.

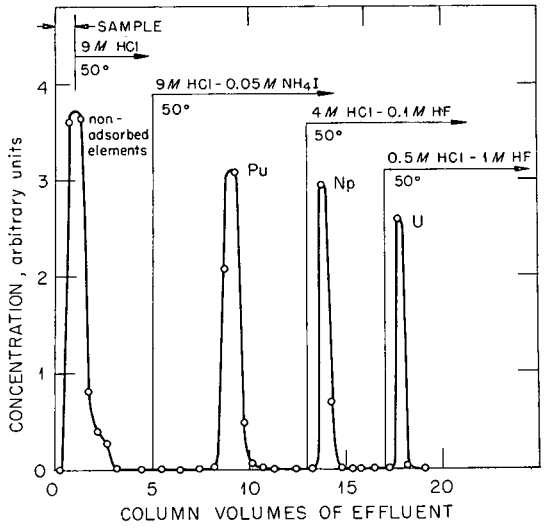


Fig. 1. Separation of Pu, Np and U (Dowex 1-X10, —400 mesh, 0.25 cm^2 × 3 cm column).

which was sufficient to remove all the non-adsorbable elements; U, Np and Pu remained adsorbed. Plutonium was removed with 9 *M* HCl–0.05 *M* NH_4I. Breakthrough of plutonium was delayed until breakthrough of iodide occurred. Neptunium was removed with 4 *M* HCl–0.1 *M* HF and uranium with 0.5 *M* HCl–1 *M* HF.

In the elution of plutonium, the HCl concentration should not exceed 10 *M*; otherwise partial reduction of U(VI) by iodide can occur and the neptunium fraction can become contaminated with uranium. For the same reason, it is probably well to avoid use of excess iodide in the eluent.

PROCEDURE

(*a*) *Materials and reagents*

Resin. Dowex 1-X10 (—400 mesh), chloride form.

Apparatus. A section of plastic tubing 0.6 cm I.D. and 12 cm in length is used to prepare the column. The tubing is pulled out to a tip at one end and a porous Teflon plug inserted to retain the resin. Additional apparatus are plastic test tubes, Teflon evaporating dishes, and plastic transfer pipettes.

Column. Resin bed: 0.28 cm^2 × 3.0 cm; column volume 0.85 ml. Flow rate: *ca.* 0.8 cm/min. Temperature: 50°.

Effluent volumes (column volumes = c.v.):

Non-adsorbed fraction: 5 c.v. (4.2 ml) of solution II.

Pu fraction: 8 c.v. (6.8 ml) of solution III.

Np fraction: 4 c.v. (3.4 ml) of solution IV.

U fraction: 3 c.v. (2.5 ml) of solution V.

Solutions. (I): 9 *M* HCl–0.05 *M* HNO_3; (II): 9 *M* HCl; (III): 9 *M* HCl–0.05 *M* NH_4I; (IV): 4 *M* HCl–0.1 *M* HF; (V): 0.5 *M* HCl–1 *M* HF.

(*b*) *Sample preparation*

The sample is dissolved by standard methods, evaporated to near dryness, and residue

is taken up in about 1 ml of 9 *M* HCl–0.05 *M* HNO_3. The solution is warmed, but not boiled, for 5 min.

(c) Column operations

Resin as a slurry in water is added to the plastic column until a resin bed about 3 cm in length (*ca.* 0.85 ml) is formed. The column is placed in a water jacket which is heated to 50°; it is then pretreated with at least 3 c.v. (2.5 ml) of 9 *M* HCl solution and the sample is added. Flow rate is controlled by air pressure to about 0.8 cm/min. After the sample has passed into the resin bed, 0.5 c.v. of 9 *M* HCl is added as wash, taking care not to disturb the resin at the top of the bed. When the wash solution has passed into the bed, an additional 3.5 c.v. (3 ml) of eluent are added and elution continued. The effluent is collected in a plastic test tube; it contains the non-adsorbed elements.

Plutonium is eluted by passing 8 c.v. (6.8 ml) of 9 *M* HCl–0.05 *M* NH_4I through the resin bed. The effluent is collected in a plastic tube. Neptunium is removed with 4 c.v. (3.4 ml) of 4 *M* HCl–0.1 *M* HF. Uranium is eluted with 0.5 *M* HCl–1 *M* HF. The column may be regenerated with 3 c.v. of 9 *M* HCl.

Column operation time for the separation of uranium, neptunium, plutonium and the non-adsorbed elements into individual fractions is about 80 min.

ACKNOWLEDGEMENT

The authors are indebted to Dr. K. A. KRAUS for valuable comments and discussion.

SUMMARY

An anion exchange procedure is described for separating uranium, neptunium and plutonium from each other and from several non-adsorbed elements.

REFERENCES

1 J. H. HOLLOWAY AND F. NELSON, *J. Chromatog.*, 14 (1964) 255.
2 D. C. HOFFMAN, in C. E. CROUTHAMEL (Editor), *Progress in Nuclear Energy*, Ser. IX, Pergamon Press, New York, N.Y., 1961, Ch. 3, p. 157.
3 K. A. KRAUS AND F. NELSON, *Proc. Intern. Conf. Peaceful Uses At. Energy, Geneva, 1955*, (United Nations, 1956), Vol. VII, pp. 113, 131.
4 E. A. C. CROUCH AND G. B. COOK, *J. Inorg. Nucl. Chem.*, 2 (1956) 223.
5 Y. MARCUS, F. NELSON, H. O. PHILLIPS AND K. A. KRAUS, *Oak Ridge Natl. Lab. Rept.*, ORNL 2584 (1958); see also, Y. MARCUS, *Israel At. Energy Comm.*, Rept. PUB-UP-R20 (1959).
6 K. A. KRAUS, G. E. MOORE AND F. NELSON, *J. Am. Chem. Soc.*, 78 (1956) 2692.

Short Communication

Über die Verwendung extrem kleinvolumiger Trennkammern in der Dünnschicht-Chromatographie

Dünnschicht-Chromatogramme wurden bisher zumeist in grossvolumigen Trennkammern entwickelt. Es besteht allgemein Einigkeit darüber, dass es notwendig ist, den Gasraum der Kammern mit dem Dampf des Lösungsmittels zu sättigen. Lässt man ein Dünnschicht-Chromatogramm ausserhalb einer Trennkammer laufen oder in einer grossvolumigen Kammer, die nicht gesättigt ist, so dampfen von den verschiedenen Zonen der Platte verschieden grosse Mengen Lösungsmittel ab. Das hat zur Folge, dass in manchen Zonen der Platte grössere Lösungsmittelmengen zur Wanderung der Substanzflecken beigetragen haben als in anderen. Folglich laufen gleiche Substanzen auf derselben Platte verschieden weit. Noch viel weniger kann man auf reproduzierbare R_F-Werte bei verschiedenen Platten hoffen. Grossvolumige Trennkammern, die für das Plattenformat 20 × 20 cm Inhalte von 2–5 l aufweisen, sättigt man gewöhnlich in der Weise, dass man einen mehr oder weniger grossen Teil der Wand innen mit Fliesspapier bedeckt, das mit dem Lösungsmittel getränkt ist. Dass auch bei diesem Vorgehen keineswegs immer eine hinreichende Sättigung erreicht wird, zeigen immer wieder beobachtete "Randeffekte". MANGOLD[1] z.B. beschreibt die Entfernung der Schicht vom Plattenrand, um dadurch den Randeffekt weitgehend auszuschalten.

In letzter Zeit sind eine Anzahl kleinvolumiger Trennkammern beschrieben worden, die alle gleichartig aufgebaut sind: die in üblicher Weise beschichtete Dünnschicht-Platte bildet die Kammerrückwand. Die Kammervorderwand wird ebenfalls durch eine Glasplatte gebildet. Um beide Wände in einem definierten, geringen Abstand voneinander zu halten, werden an den Seitenkanten, meist auch an der Oberkante, Distanzstücke vorgesehen. Dort, wo die Distanzstücke mit der Dünnschicht-Platte in Berührung kommen, wird die Sorptionsmittelschicht zuvor entfernt. Diese schmale, unten offene Kammer, die in allen Fällen durch irgendwelche Klammern zusammengehalten wird, taucht man in einen Trog, der das Lösungsmittel enthält.

1. STAHL[2] beschreibt 1962 das sogenannte "S-Kammer-System". Distanzstücke und Vorderwand der Kammer bilden hier eine Einheit, indem die sogenannte Deckplatte aufgeschmolzene Glasstreifen trägt, die oberflächlich plangeschliffen sind.

2 Ende 1962 benutzt HONEGGER[3] ein gleichartiges, selbstgebautes System, bei dem die Glasstreifen lediglich nicht aufgeschmolzen sind.

3. WASICKY[4] beschreibt im April 1963 wiederum ein derartiges System, bei dem als Distanzelement ein "weicher Draht" verwendet wird, und gleichzeitig beschreibt DAVIES[5] eine solche Anordnung mit dem Unterschied, dass ein gebogener Glasstab als Distanzstück verwendet wird.

4. Kurz davor brachten wir ein System heraus, bei dem als Distanzstück ein Papprahmen verwendet wird. Zuvor hatten wir Versuche angestellt, als Distanzelement Teflon zu verwenden, doch zeigte sich der weiche, leicht dichtende Pappstreifen allen anderen Dichtungselementen überlegen, selbst den Glasstreifen, da der geringste Rest Sorptionsmittel auf dem freigelegten Streifen ein Dichtschliessen unmöglich macht.

Dieses Kammersystem, das kommerziell erhältlich ist*, nannten wir seines Aufbaues wegen "Sandwich-Kammer". Fig. 1 zeigt das Schema, in der Fig. 2 sieht man die Kammer in Betrieb. Die eigentliche Dünnschicht-Platte (1) mit den aufgetragenen Substanzen ist an drei Seiten vom Sorptionsmittel befreit. Darauf wird der Papprahmen (2) gelegt und schliesslich eine Glasplatte (3) darüber plaziert. Das Ganze wird mit den Federklammern (4) zusammengehalten, von denen zwei an dem Ständer (5) befestigt sind. Dieser "Sandwich" taucht mit seiner Unterkante in den Lösungsmitteltrog (6).

In manchen Fällen ist es nicht zulässig, einen offenen Lösungsmitteltrog zu verwenden, da heterogene Lösungsmittel während der Laufzeit des Chromatogramms ihre Zusammensetzung ändern können. Es ist dann empfehlenswert, den Sandwich in eine herkömmliche Entwicklungskammer zu stellen, die ein selektives Abdampfen einer Lösungsmittelkomponente verhindert.

Sofern man nicht wünscht, den Pappstreifen in das Lösungsmittel eintauchen zu lassen, kann man ihn um einige Millimeter kürzen, sodass er dicht über der Oberfläche des Lösungsmittels beginnt.

Der Vorteil dieser extrem dünnen Kammern, im folgenden der Einfachheit halber alle "Sandwichkammern" genannt, ist der, dass zur Sättigung des Gasraumes nur sehr wenig Lösungsmittel gebraucht wird. Man kann es also der aufsteigenden Front entziehen, ohne die eingangs geschilderten Unzuträglichkeiten in Kauf nehmen zu müssen. Beispielsweise wird zur Sättigung der CAMAG-Sandwichkammer nur etwa der 150. Teil der Lösungsmittelmenge benötigt, die in einer herkömmlichen Trennkammer verdampft werden muss. Aus diesem Grunde sprechen auch alle vorgenannten Autoren[2,4,5] davon, dass man sich um die Sättigung des Kammervolumens bei dem Sandwich-System nicht zu kümmern braucht. Lediglich HONEGGER[3] wies schon darauf hin, dass es mitunter einen Vorteil bietet, die Innenseite der Deckplatte mit lösungsmittelgetränktem Fliesspapier zu bedecken.

Unsere eigenen Versuche haben nun gezeigt, dass es in vielen Fällen unerlässlich ist, auch Sandwichkammer-Systeme vor dem Entwickeln des Chromatogramms mit Lösungsmitteldampf zu sättigen. Unterlässt man diese Sättigung, so zeigen sich dann Störungen, wenn man heterogene Lösungsmittel verwendet, deren Komponenten sich hinsichtlich ihrer Dampfdichten stark unterscheiden. In solchen Fällen treten die typischen Entmischungserscheinungen auf, d.h. man hat zwei oder mehrere Lösungsmittelfronten auf dem Chromatogramm.

Zwei Beispiele, die für diese Störung typisch sind, sollen zur Erläuterung dien. Wir benutzten ein Gemisch bestehend aus 98 % Chloroform und 2 % Methanol. Mit diesem Gemisch sollten Steroidhormone auf Kieselgel getrennt werden. Nachdem das Chromatogramm entwickelt war und unter der U.V.-Lampe begutachtet wurde, zeigte sich, dass nach 30 mm Laufstrecke offenbar nur noch reines Chloroform ge-

* CAMAG, Homburgerstrasse 24, Muttenz, Schweiz.

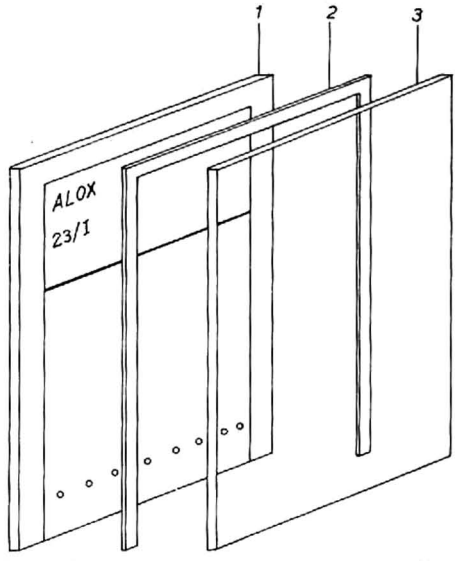


Fig. 1. Sandwichkammer, schematische Aufbau.

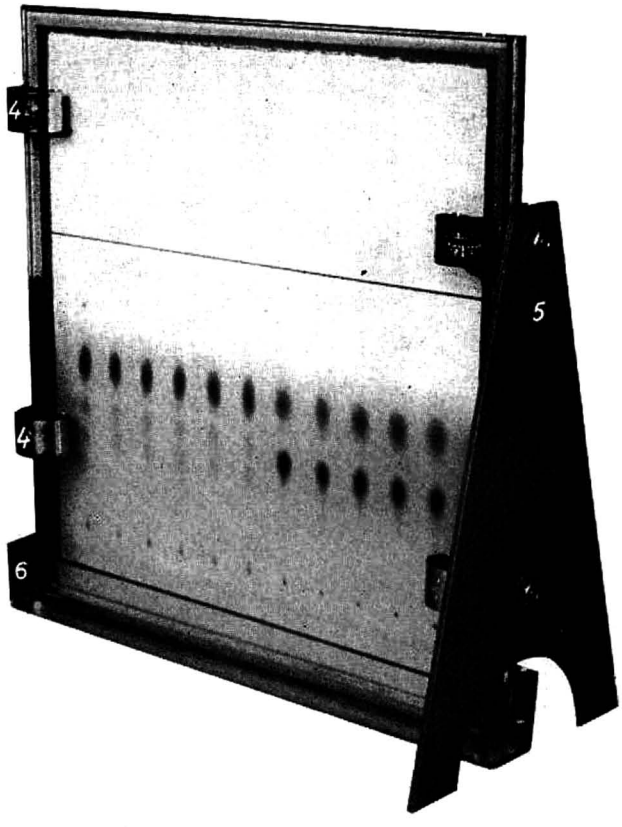


Fig. 2. Sandwichkammer in Betrieb.

laufen war. In einem anderen Fall benutzten wir als Laufmittel ein Chloroform–Methanol Gemisch (80:20). Hier trat die Entmischung erst bei 80 mm Laufstrecke auf.

Dieses Phänomen kann man wie folgt erklären: die Sättigung des Gasraumes erfolgt bei diesem Sandwich-System ausschliesslich durch Abdampfen von Lösungsmittel von der aufsteigenden Front. Da jedoch Chloroformdampf wesentlich schwerer ist als der Dampf des Methanols, sinkt ersterer zu Boden, und der obere Teil der Kammer füllt sich vorzugsweise mit Methanoldampf. Je kleiner der Anteil des Methanols ist, umso schneller verarmt die aufsteigende Front vollständig an diesem Lösungsmittel, und es steigt nur noch reines Chloroform auf. Da in einem so schmalen Raum die normale Konvektion stark beschränkt ist, tritt auch kaum erneute Mischung der Dämpfe im Gasraum ein.

Diese Annahme wird bestätigt durch die Art, wie man dieser Störung abhelfen kann: verwendet man als Deckplatte eine beschichtete Dünnschicht-Platte, von der das Sorptionsmittel an drei Seiten abgestreift wurde, und tränkt man diese Schicht mit dem Lösungsmittel, das zum Entwickeln des Chromatogramms benutzt werden soll, so besteht schon eine ideale Sättigung des Gasraumes, bevor das Chromatogramm zu laufen beginnt. Störungen der vorgenannten Art treten nicht mehr auf.

Als weiterer Vorteil ist zu vermerken, dass die R_F-Werte sehr gut reproduzierbar sind. Gegenüber einem Fliesspapier hat die Verwendung einer beschichteten Platte den Vorteil, dass mit Sicherheit eine Berührung zwischen ihr und der eigentlichen Chromatographie-Platte vermieden wird.

Selbstverständlich ist es nicht nötig, auf der Deckplatte das gleiche Sorptionsmittel zu verwenden, das als Chromatographie-Schicht vorgesehen ist. Empfehlenswert ist es, für diesen Zweck die mechanisch sehr widerstandsfähigen Celluloseschichten zu verwenden.

CAMAG, Muttenz (Schweiz) D. JÄNCHEN

[1] H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.
[2] E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, Göttingen, Heidelberg, 1962.
[3] C. G. HONEGGER, Privatmitteilung.
[4] R. WASICKY, *Naturwiss.*, 50 (1963) 569.
[5] B. H. DAVIES, *J. Chromatog.*, 10 (1963) 518.

Eingegangen den 25. November 1963

Notes

Thin-layer chromatography under controlled conditions

Within the past few years thin-layer chromatography (TLC) has developed into an important chromatographic technique. Although TLC has many advantages, the fact that it is, in principle, chromatography with "open" columns gives rise to a number of problems which cannot be solved without special precautions and suitable equipment.

One of these problems is caused by atmospheric humidity which can influence separation on chromatoplates. Though this phenomenon is now fairly well known, its effect can be more disadvantageous than hitherto recognized. In our work on the separation of 2,4-DNPs* of aliphatic carbonyl compounds into classes[1,2] it was found that although an oven-dried plate was used even the humidity of the air in a chromatoplate jar was sufficient to prevent separation. Separation could only be obtained when a chromatoplate jar was used in which it was possible to work in complete absence of atmospheric humidity. Results are given in Figs. 1A and 1B.

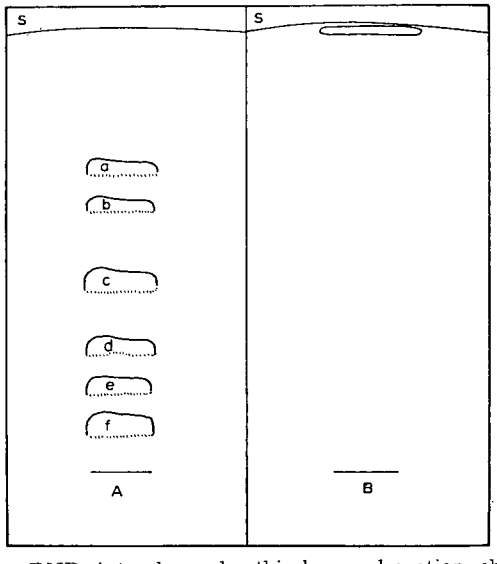


Fig. 1. Separation of 2,4-DNPs into classes by thin-layer adsorption chromatography on basic zinc carbonate. Mobile phase petroleum ether bp. 60–70°–benzene–pyridine (7:1:2) containing 0.2% abs. ethanol. A. Chromatography in absence of water vapour: a = hexanone-2; b = pentanone-3; c = heptanal; d = hept-2-enone-4; e = oct-2-enal; g = deca-2,4-dienal; s = solvent front; plate 8 × 18 cm. B. Chromatography in presence of water vapour. The same mixture of DNPs as in (A) is not resolved and moves close to the solvent front.

* 2,4-Dinitrophenylhydrazones.

Another important source of problems is the presence of oxygen during the chromatography of lipids. In TLC the risk of autoxidation of lipids is even greater than in column chromatography. To demonstrate this effect, a mixture of fatty acid methyl esters was run on two chromatoplates, the first in an atmosphere of nitrogen and the second in air. The methyl esters were extracted from the two adsorbent layers and the fatty acid composition was determined by gas chromatography. Results are given in Table I. It can be seen that there is a significant decrease in the amount of higher unsaturated fatty acids as a result of autoxidation.

In order to overcome the above problems we designed the chromatoplate chamber shown in Fig. 2. It consists of a glass tank (20 × 20 × 5 cm inner dimensions) closed by a glass plate with a hole to which a female joint was cemented. In one side of the jar an opening was drilled for the introduction of the mobile phase from a funnel fastened to the side of the chamber. To obtain an air-tight seal with the covering plate, some silicone grease was applied to the polished rim of the tank.

TABLE I

INFLUENCE OF OXYGEN (DURING TLC) ON THE COMPOSITION OF A MIXTURE OF FATTY ACID METHYL ESTERS

The figures refer to peak areas of fatty acid methyl esters separated by TLC relative to methyl stearate (= 100).

	Composition of the mixture of fatty acid methyl esters		
	before TLC	*after TLC in N_2*	*after TLC in air*
Methyl stearate	100	100	100
Methyl oleate	31	31	31
Methyl linoleate	92	91	87
Methyl linolenate	115	115	108
Methyl arachidonate	82	83	72

When carrying out a separation, the spotted plate was placed in the chamber (with a piece of filter paper covering the walls of the jar if the chamber had to be saturated with the solvent vapour). The lid was closed and, using the two openings in the chamber, dried nitrogen (or air) was passed through the chamber. After displacement of moisture (and oxygen) the mobile phase was introduced while the chamber was tilted backwards in order to wet the piece of filter paper. When the atmosphere in the jar was saturated with vapour, the jar was placed in a vertical position so as to bring the plate in contact with the mobile phase which began to ascend. The development of too high a pressure in the chamber was prevented by opening the tap at the side of the funnel.

Using the equipment described, it was possible to prevent the undesirable effects of humidity and of oxygen. R_F values were very reproducible as can be seen in Fig. 3, *e.g.*, where the results of separation of 2,4-DNPs according to chain length[2] are given.

It was also possible to carry out separations on chromatoplates of lower activity by leading air (or nitrogen) of fixed humidity through the chromatoplate chamber.

In our experiments plates of different size were coated with a thin layer of ad-

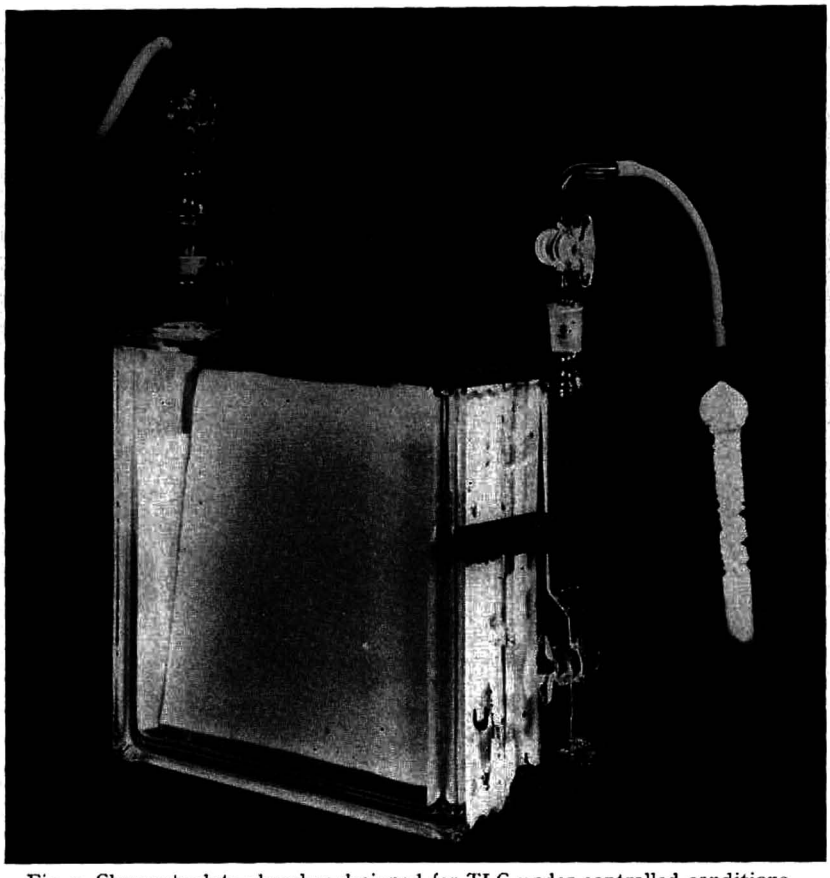


Fig. 2. Chromatoplate chamber designed for TLC under controlled conditions.

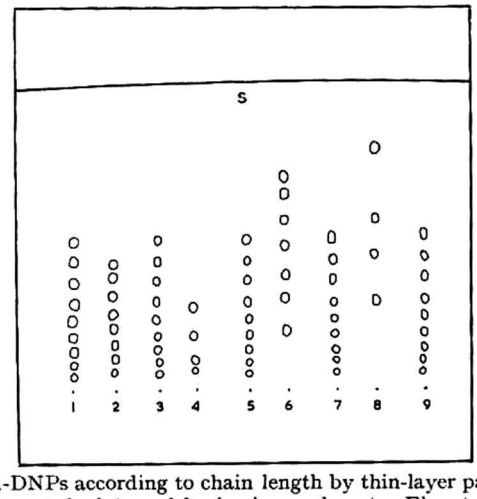


Fig. 3. Separation of 2,4-DNPs according to chain length by thin-layer partition chromatography on Carbowax-400 impregnated plates of basic zinc carbonate. Eluent = petroleum ether, bp. 100–120°. 1 = alkanals C_2 to C_{10} incl.; 2 = alk-2-enals C_4 to C_{11} incl.; 3 = same as in 1; 4 = alka-2,4-dienals C_6, C_7, C_9, C_{11}; 5 = same as in 1; 6 = alkanones-2 C_3 to C_8 incl.; 7 = same as 1; 8 = alk-2-enones-4 C_5, C_6, C_7, C_{10}; 9 = same as 1; s = solvent front; plate 15 × 15 cm.

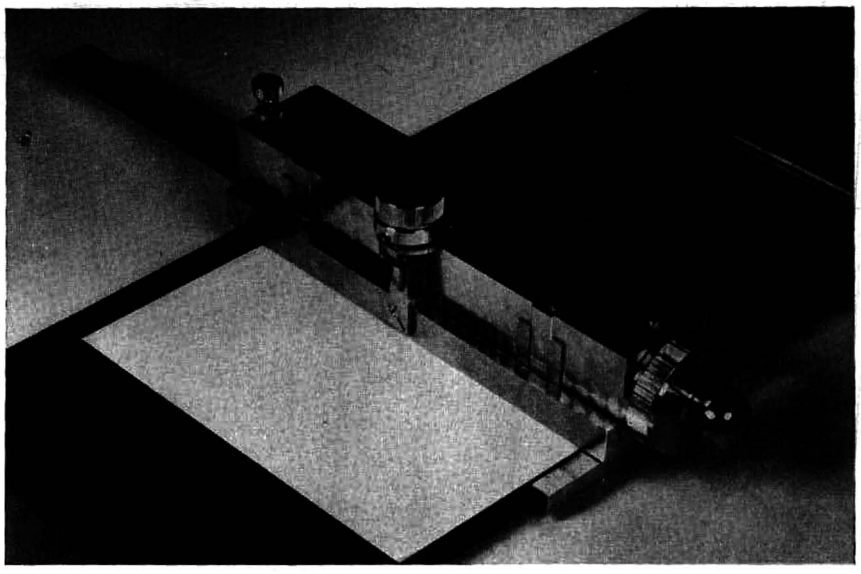


Fig. 4. Applicator for thin-layer chromatography.

sorbent using an applicator of our own design. With this applicator, shown in Fig. 4, a layer of the desired thickness (max. 2 mm) can be applied to plates of different widths. The slide on the left side of the applicator is extended according to the width of the plate, while the slide at the front of the instrument is moved vertically to adjust the adsorbent layer to the desired thickness.

I wish to thank Mr. C. DE GRAAF and Mr. A. M. BAKKENES for carrying out the construction of the designed chromatoplate chamber and the applicator respectively.

Netherlands Institute for Dairy Research (NIZO),
Ede (The Netherlands)
H. T. BADINGS

[1] H. T. BADINGS, *J. Am. Oil Chemists' Soc.*, 36 (1959) 648.
[2] H. T. BADINGS AND J. G. WASSINK, *Neth. Milk Dairy J.*, 17 (1963) 132.

Received September 20th, 1963

Thin-layer chromatography of steroids: Specificity of two location reagents

Although there are many location reagents that can be used for detecting steroids, there are few of known specificity. When the specificity has been determined, it has been found that several of these reagents are either of limited application, *e.g.* isonicotinic acid hydrazide for Δ^4- and $\Delta^{1,4}$-3-keto steroids, or of low sensitivity, *e.g.* 2,4-dinitrophenyl-hydrazine (+ hydrochloric acid) for ketones.

During routine examination by thin-layer chromatography of compounds produced during the synthesis of corticosteroids from sapogenins and of synthetic C_{21} and C_{22} intermediates (the latter containing a 16-methyl or -methylene group), we have found three location reagents adequate to deal with almost all samples, namely, 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride (T.P.T.Z.) (M & B 1767), effective on alumina, *p*-hydroxybenzaldehyde–sulphuric acid (Komarowsky's reagent)[1] and methanolic zinc chloride; the last two were studied mainly on silica gel, but were effective also on alumina. A brief note mentioning this work has already been published[6].

The tetrazolium salts are well known for use in the detection of reducing steroids; we mention the styryl derivative because in our view it is an improvement on the more usual anisole derivative. Its use has already been reported, but not perhaps sufficiently stressed[2]. This styryl phenyl derivative produces strong purple spots on a yellow ground, visible in daylight, compared with the weaker lilac spots given by the dianisole derivative ("blue tetrazolium") commonly used.

Use of Komarowsky's reagent was reported for location of sapogenins[3], but it was subsequently found that 23-substituted sapogenins would not react. The 23-position can be regarded as α to a potential C_{22} ketone, by considering the spiroketal group as the internal ketal of a 16,26-dihydroxy-22-ketone, and it is thought that *p*-hydroxybenzaldehyde condenses at the 23-position in the presence of sulphuric acid. Komarowsky's reagent has also been used for the detection of several corticosteroids[5]. More detailed examination has shown that it will react with all 3-keto steroids having an unsubstituted C_2 position, which is the active site α to the 3-ketone*. The reaction is thus analogous to that for sapogenins, both requiring a methylene group α to a ketone. Spots are visible in daylight and are generally yellow on a white ground, which will gradually turn pink if the plate has been heated at 100°.

Zinc chloride is, in our view, greatly to be preferred to the most commonly used metal halide, antimony trichloride, especially since the latter gives best results when used in conjunction with both thionyl chloride and chlorine[4]. Zinc chloride is non-corrosive and virtually non-toxic, the only precaution required in handling it being to protect it from moisture, as it is highly deliquescent. There are no unpleasant fumes produced by it during either spraying or heating of the plate.

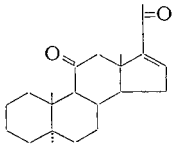
The specificity of zinc chloride for C_{21} and C_{22} steroids can be summarised as follows. All 3-hydroxy or 11-hydroxy derivatives are located, but for 3-ketones some form of activation is required, notably by an epoxide or a double bond. The activating effect of more distant groups, such as a 16,17 epoxide, is observed only when there is

* Substitution of the 3-ketone does not affect sensitivity, but this is diminished by substitution of the C_4 position, for example by a double bond or bromine atom.

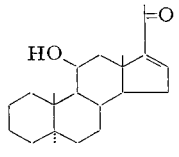
no 11-ketone in ring C. In the absence of an oxygen function at C_3, only 11-hydroxy derivatives will react.

Location of 3-ketones was achieved in the presence of 9,11- and 16,17-epoxides, of 11-hydroxyl groups, and of $\Delta^{1:4}$-, $\Delta^{4:6}$-, and $\Delta^{9:16}$- double bonds. The only effective monoenes were the $\Delta^{9(11)}$-derivatives, whereas Δ^{1}-, Δ^{4}- and Δ^{16}-3-keto steroids showed weak reactions.

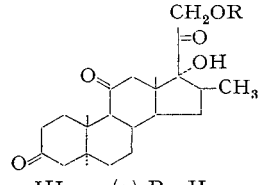
A double bond or an epoxide grouping can presumably produce reactivity at the 3-keto position because the steroid ring structure is under strain. In 3-keto-11-hydroxy compounds there is no distortion of the rings, and the reaction occurs at the 11 position. This was demonstrated by converting 5α-pregnan-16-ene-11,20-dione (I), a 3-desoxy compound not located by zinc chloride, to the corresponding 11-hydroxy derivative (II), which was then easily detectable.



I



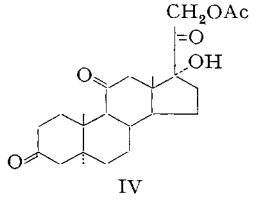
II



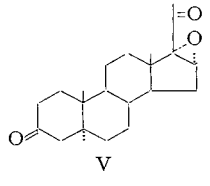
III (a) R=H
(b) R=CH_3CO

The presence of a 16-methyl group does not improve reactivity, since zinc chloride will not locate 16β-methyl-4,5α-dihydrocortisone 21-alcohol or acetate (III a + b), two C_{22} 3-keto steroids that do not contain a double bond or an epoxide grouping. The corresponding C_{21} steroid, 4,5α-dihydrocortisone acetate (IV), also gives weak spots with zinc chloride.

The deactivating effect of an 11-ketone is shown by 16α,17-oxido-5α-pregnane-3,20-dione (V), which gives strong spots with zinc chloride, whereas the introduction of an 11-ketone produces a nonreacting compound.



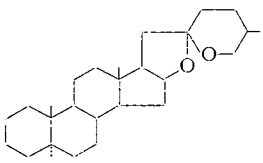
IV

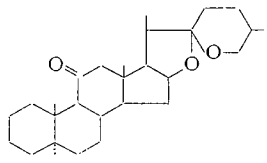


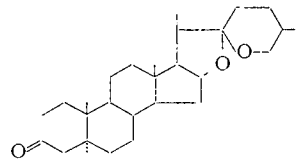
V

In steroidal sapogenins there is no specific group that appears necessary, and the reactivity could well be intrinsic to the spirostane system, since a compound containing no substituent groups, 5α,25D-spirostane (VI), is still located with zinc chloride. All sapogenins examined were found to react, including 3-desoxy compounds, such as 5α,25D-spirostan-11-one (VII), and 3-ketones, such as 5α,25D-spirostan-3-one (VIII) ("tigogenone").

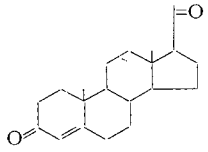
The variety of colours produced makes this reagent particularly useful for the examination of mixtures of close-running spots. Some spots are visible in daylight, but all reacting steroids give vivid spots of various colours when seen under U.V. light of 366 mμ wavelength.

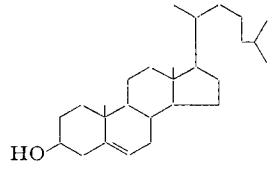

VI


VII

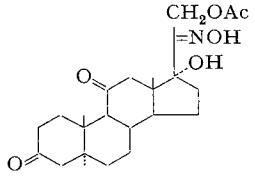

VIII

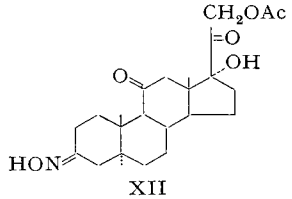
It should be stressed that the presence of a 3-hydroxy group does not apparently guarantee the location of all classes of steroid, for example sterols. Thus the only reference to the use of methanolic zinc chloride[5], which relates to its application for paper chromatography, claims positive detection of several oestrogens and androgens but not of progesterone (IX), a 3-keto steroid, or of cholesterol (X), a 3β-hydroxy steroid. We have not had the opportunity to examine such compounds by thin-layer chromatography.


IX


X

In the course of examining over 250 different steroids restricted to the classes mentioned initially we have only come across one anomalous reaction, that of the 3- and 20-oxime groups. The 20-oxime of 4,5α-dihydrocortisone acetate (XI) gives positive results with zinc chloride, and the 3-oxime (XII) is not detected. Neither would have been expected to react, since the parent compound (IV) does not. It would appear that the 20-oxime group is reacting as an hydroxyl compound rather than as a substituted ketone, but there is no obvious reason why only the 20 position should react in this way, except that the side chain is more easily able than ring A to undergo rearrangements.


XI


XII

It must be emphasised both for Komarowsky's reagent and for zinc chloride that faint colours are produced with a number of so-called unreacting compounds. However, the sensitivity is greatly diminished for these steroids, whereas for all three location reagents the detection limit for reacting steroids is 0.1 μg or less. It is therefore possible, with a 1 % solution and 2.5 λ loading, to detect 1 % or less of an impurity.

Preparation of location reagents

2,5-Diphenyl-3-(4-styrylphenyl)-tetrazolium chloride. Mix 1 part of a 1% solution of

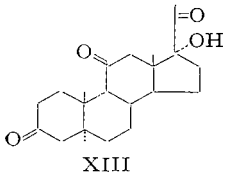
T.P.T.Z. salt in methanol with 10 parts of a 3 % solution of sodium hydroxide in distilled water, and apply immediately.

Komarowsky's reagent. Mix 1 part of a 50 % v/v solution of concentrated sulphuric acid in distilled water with 10 parts of a 2 % solution of *p*-hydroxybenzaldehyde in methanol. Apply immediately, and heat the sprayed plate for 3–4 min at 105° or 10 min at 60° (less background colour develops).

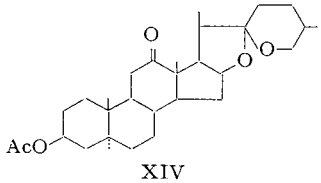
Zinc chloride. Prepare a 30 % solution of technical grade zinc chloride in analytical reagent grade methanol, and filter the cloudy solution through a sterimat. After spraying, heat the plate for 60 min at 105°, and cover immediately with a second plate on removing the chromatoplate from the oven (the intensity and colour of spots is rapidly affected by atmospheric moisture). Examine under U.V. light of 366 mμ wavelength.

TABLE I

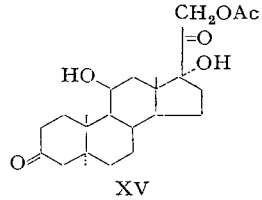
Formula	Basic no. of C atoms	Steroid (trival name)	Characteristic groups				Location reagent		
			3	11	17	21	Komarowsky's	Zinc-chlor.	T.P.T.Z.
XIII	21	21-Desoxydihydrocortisone	CO	CO	OH	CH_3	+	—	—
XIV	27	Hecogenin acetate	OAc	—	—	—	+	+	—
XV	21	Dihydrocortisol acetate	CO	OH	OH	OAc	+	+	+
IV	21	Dihydrocortisone acetate	CO	CO	OH	OAc	+	—	+
XVI	21	Prednisone acetate	CO	CO	OH	OAc	—	+	+
XVII	21	3,17-Desoxydihydrocortisone	—	CO	—	OH	—	—	+
XVIII	21	3,17,21-Desoxydihydrocortisone	—	CO	—	CH_3	—	—	—
XIX	27	11,23-Dibromohecogenin acetate	OAc	Br	—	—	—	+	—



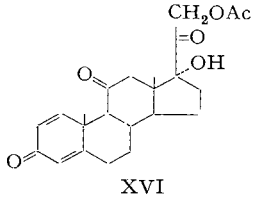
XIII



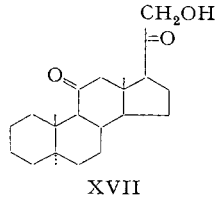
XIV



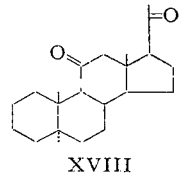
XV



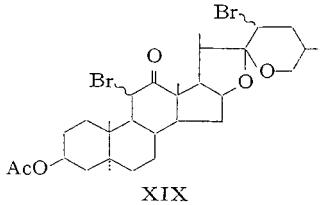
XVI



XVII



XVIII



XIX

Examples of differently reacting steroids

Some examples of the use of the above location reagents are given in Table I.

Note added in proof

The tetrazolium reagent is also effective on silica gel, provided that an 8% (2 *N*) solution of sodium hydroxide is used to ensure alkaline conditions on the adsorbent.

Glaxo Laboratories Ltd., Montrose, Angus PETER J. STEVENS
(*Great Britain*)

[1] S. D. NOGARE AND J. MITCHELL, *Anal. Chem.*, 25 (1953) 1376.
[2] S. G. BROOKS, R. M. EVANS, G. F. H. GREEN, J. S. HUNT, A. G. LONG, B. MOONEY AND L. J. WYMAN, *J. Chem. Soc.*, (1958) 4614.
[3] R. K. CALLOW, D. H. W. DICKSON, J. ELKS, R. M. EVANS, V. H. T. JAMES, A. G. LONG, J. F. OUGHTON AND J. E. PAGE, *J. Chem. Soc.*, (1955) 1966.
[4] J. KUČERA, Ž. PROCHÁZKA AND K. VEREŠ, *Collection Czechoslov. Chem. Communs.*, 22 (1957) 1185.
[5] J. F. NYC, J. B. GARST, H. B. FRIEDGOOD AND D. M. MARON, *Arch. Biochem.*, 29 (1950) 219.
[6] P. J. STEVENS, *Proc. Assoc. Clin. Biochem.*, 2 (1963) 156.

Received August 23rd, 1963

J. Chromatog., 14 (1964) 269–273

Reverse phase thin-layer chromatography of 2,4-dinitrophenylhydrazones of *n*-alkanals and *n*-alkan-2-ones*

URBACH[1] of C.S.I.R.O. (Melbourne) has recently published a comprehensive thin-layer chromatographic (TLC) study of the 2,4-dinitrophenylhydrazones (DNPH's) of the aliphatic monocarbonyls. The author briefly reviewed the pertinent literature, and presented methods for both class and homolog separations. Our purpose in this communication is to briefly describe an alternate method for the separation by TLC of an homologous series of the DNPH's of aliphatic aldehydes and ketones. In URBACH's system, chain-length separation is achieved on Kieselguhr G plates impregnated with 2-phenoxyethanol; the impregnated plates are spotted with derivatives, and developed several times with 4% diethyl ether in light petroleum. Resolution of the DNPH's of C_1–C_{14} *n*-alkanals and C_3–C_{13} *n*-alkan-2-ones was achieved; the higher members of the series moved the fastest.

In our method we have adapted the KLEIN AND DE JONG[2] paper chromatographic procedure for TLC. Glass plates 5 × 20 cm were coated with a 250 μ layer of silica gel G using the Brinkmann apparatus. After allowing about 15 min for the adsorbent to set, the plates were placed in an oven and heated at 110° for at least 1 h. The plates were then cooled to room temperature and very slowly immersed in petroleum ether (boiling range 30–60°) containing 10% (v/v) of Shell Ondina 27 mineral oil. After impregnation, the petroleum ether was allowed to evaporate at room temperature, and the plates were then spotted. The plates were given a single development with

* This investigation was supported by Public Health Service Research Grant EF-269 from the Division of Environmental Engineering and Food Protection.

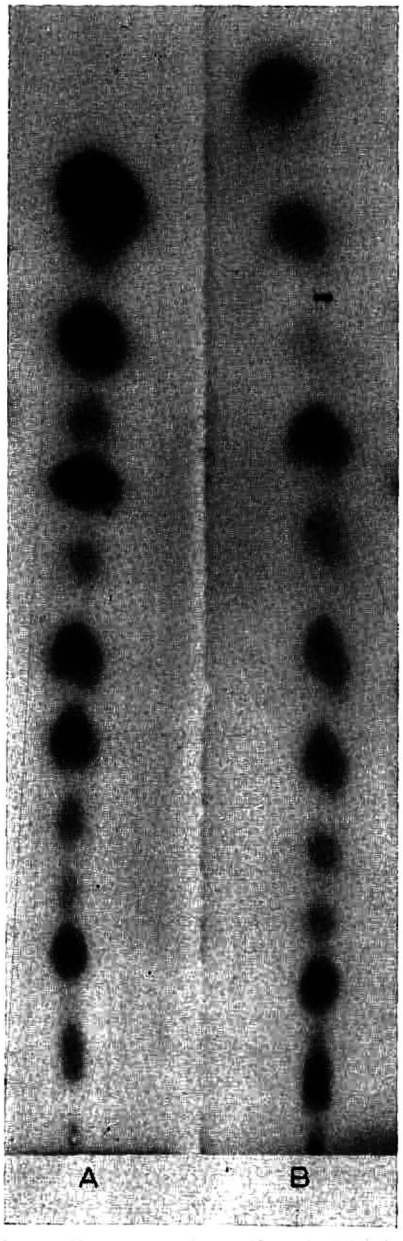


Fig. 1. TLC of C_1–C_{14} *n*-alkanal DNPH's (A and B are from two different runs). Developed for 7 h with dioxane–water (65:35, v/v) using the ordinary TLC cylinder as a chromatographic chamber. The C_1 derivative moved the fastest, the C_{14} the slowest.

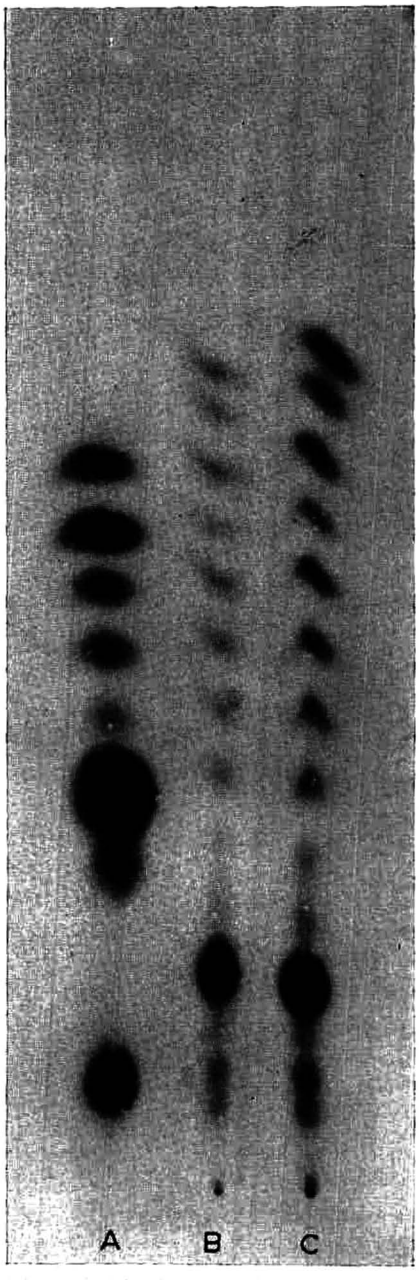


Fig. 2. TLC using "continuous development". Developed for 7 h with dioxane–water (65:35, v/v) in a 1000 ml beaker covered with Saran film; 3–4 cm of the top of the plate was exposed to the atmosphere through a slit in the film. (A) C_3–$C_{9,13}$ *n*-alkan-2-one DNPH's. (B) and (C) C_1–C_{14} *n*-alkanal DNPH's.

dioxane–water (65:35, v/v). A single development took about 6 h at 25°; multiple development, in this particular system, did not result in further resolution. Fig. 1 shows a photograph, taken under U.V. light, of the TLC of the C_1–C_{14} *n*-alkanal DNPH's using the technique described. We have observed that the best separation is achieved with the middle members of the C_1–C_{14} series; the C_1 and C_2 derivatives tend to run together, and the C_{13} and C_{14} derivatives are often not well separated.

Although multiple development did not enhance resolution, we have used what we call "continuous development" to increase resolution. In this technique the derivatives are spotted into the impregnated plates, and the plates are developed with the dioxane–water system with 3–4 cm of the top of the plates exposed to the atmosphere. This is conveniently done in a Saran*-covered 1000 ml beaker, with a slit cut in the Saran film for the plate. Using this technique the slow and medium-mobility fractions are usually well resolved. Overdevelopment, however, can cause the "piling-up" of the fast-moving fractions at the top of the plate. Fig. 2 shows a plate that had been run with "continuous development".

Department of Food Science and Technology,
Oregon State University,
Corvallis, Oreg. (U.S.A.)

L. M. LIBBEY
E. A. DAY

[1] G. URBACH, *J. Chromatog.*, 12 (1963) 196.
[2] F. KLEIN AND K. DE JONG, *Rec. Trav. Chim.*, 75 (1956) 1285.

Received September 12th, 1963

* Saran is a trade name for polyvinylidene chloride.

Thin-layer chromatography of tetra- and pentacyclic triterpenes

Thin-layer chromatography has occasionally been applied to the separation of triterpenes, *e.g.* by TSCHESCHE[1,2] to those of *Bredemeyera floribunda*, by THOMAS[3] to those of *Commiphora glandulosa* and by HUNECK[4] to those of *Sorbus torminalis*.

In experiments with Israeli peat, which will be reported elsewhere, we have developed a system that proved useful in the separation of triterpenoid compounds and permitted their easy identification. The solvent mixture used was heptane–benzene–ethanol (50:50:0.5), applied to alumina G. This mixture has the advantage that an increase in the alcohol concentration increases and a decrease in its concentration decreases the rate of migration. For example, the R_F values for betulin (No. 3) are 0, 0.14, 0.73 for 0 %, 0.5 % and 2 % alcohol, for lupeol (No. 10) 0.16, 0.37, 0.94 for the same three alcohol concentrations.

A systematic study has given the following results, which will be extended by further investigations: *epi*-β-Amyrin (No. 15) and *epi*-lupeol (14) can be separated from their diastereoisomers β-amyrin (No. 6) and lupeol (10); the *epi*-compounds have higher R_F values.

Friedelin (19) can be easily separated from friedelan-3β-ol (13), euphone (31) from euphol (24), allobetulone (17) from allobetulin (11). In these cases, the ketones have higher R_F values than the corresponding secondary alcohols. Equally, the esters of alcohols have higher R_F values than the free alcohols.

In Tables I and II are listed the tetra- and pentacyclic triterpenes so far studied, together with their R_F values and the colours obtained by spraying with three reagents. For convenience, the structural formulae of the compounds investigated are given in Fig. 1. In the tables, the compounds are arranged in the order of increasing R_F.

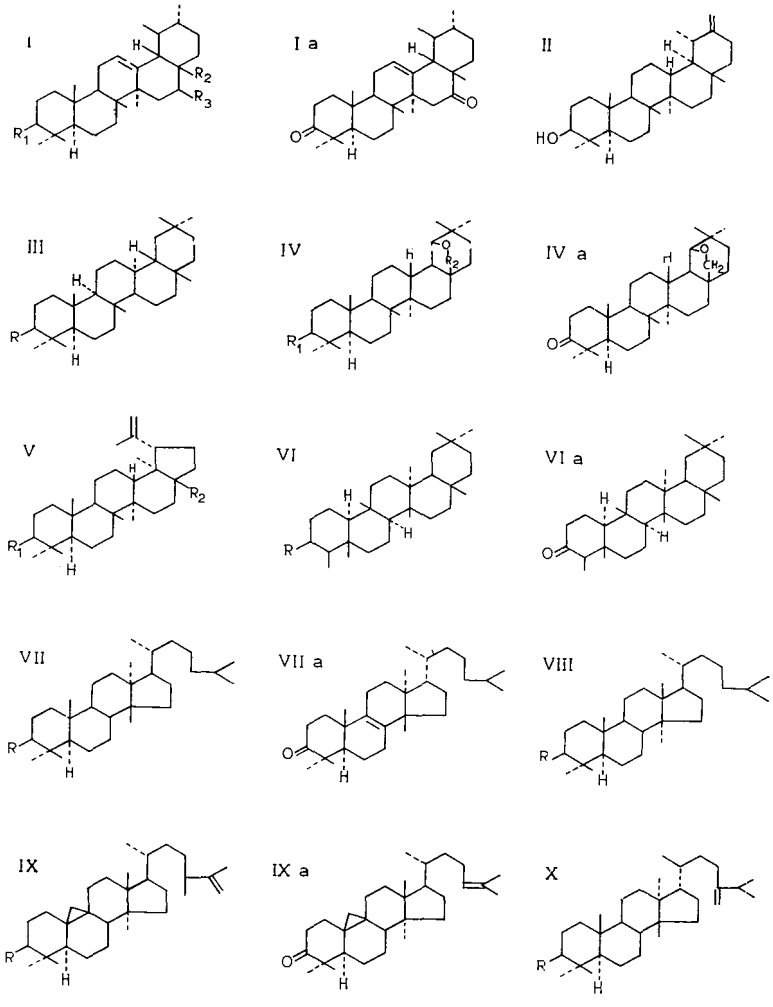


Fig. 1.

In the α-amyrin (I) series, the R_F value decreases with the increasing number of hydroxyl groups. This is also evident in other series, *e.g.* (V). For the β-amyrin (III) compounds, the double bond has a small, but significant effect. If R = OH, the R_F

TABLE I

PENTACYCLIC TRITERPENES

No.	*Compound*	*Structure*	*Substituents*	R_F	$SbCl_3$	$SbCl_5$	Ac_2O/H_2SO_4
1	Uvaol	I	$R_1 = OH$, $R_2 = CH_2OH$, $R_3 = H$	0.03	Blue	Violet	Violet
2	Brein	I	$R_1 = OH$, $R_2 = CH_3$, $R_3 = OH$	0.04	Blue-yellow	Gray	Blue
3	Betulin	V	$R_1 = OH$, $R_2 = CH_2OH$	0.14	Violet	Pink	Violet
4	Taraxasterol	II		0.15	Violet-gray	Violet	Pink
5	Germanicol	III	R = OH, Δ18,19	0.17	Violet	Violet	Pink
6	β-Amyrin	III	R = OH, Δ12,13	0.24	Brown-gray	Pink	Pink
7	α-Amyrin	I	$R_1 = OH$, $R_2 = CH_3$, $R_3 = H$	0.26	Brown-orange	Pink	Pink
8	Taraxerol	III	R = OH, Δ14,15	0.30	Gray	Violet	Pink
9	Dihydrotaraxerol	III	R = OH	0.35	Gray-violet	Brown	Pale brown
10	Lupeol	V	$R_1 = OH$, $R_2 = CH_3$	0.37	Violet-orange	Violet	Violet
11	Allobetulin	IV	$R_1 = OH$, $R_2 = CH_2$	0.38	Yellow	Brown	Yellow
12	Breindione	Ia		0.40	Brown	Yellow	Pink
13	Friedelan-3β-ol	VI	R = OH	0.50	Violet	Violet	Pale brown
14	*epi*-Lupeol	V	$R_1 = \alpha OH$, $R_2 = CH_3$	0.52	Brown	Violet	Brown
15	*epi*-β-Amyrin	III	R = αOH, Δ12,13	0.53	Brown-gray	Violet	Pale brown
16	Oxyallobetulin	IV	$R_1 = OH$, $R_2 = (C = O)$	0.55	Pale brown	Brown	Yellow
17	Allobetulone	IVa		0.78	Pale brown	Brown	Yellow brown
18	β-Amyrin acetate	III	R = OAc, Δ12,13	0.87	Brown	Violet	Violet
19	Friedelin	VIa		0.88	Brown	Brown	Pale brown
20	*epi*-Friedelanyl acetate	VI	R = OAc	0.89	Violet	Brown	Pale brown
21	Taraxeryl acetate	III	R = OAc, Δ14,15	0.91	Gray	Violet	Violet
22	Allobetulin acetate	IV	$R_1 = OAc$, $R_2 = CH_2$	0.91	Violet	Brown	Pale brown
23	β-Amyrin benzoate	III	R = OBz, Δ12,13	0.92	Brown-orange	Violet	Brown

TABLE II

TETRACYCLIC TRITERPENES

No.	*Compound*	*Structure*	*Substituents*	R_F	$SbCl_3$	$SbCl_5$	Ac_2O/H_2SO_4
24	Euphol	VII	R = OH, Δ8,9, Δ24,25	0.20	Brown	Brown	Violet-gray
25	Parkeol	VII	R = OH, Δ9,11, Δ24,25	0.20	Brown	Brown	Violet-gray
26	Cyclolaudenol	IX	R = OH	0.21	Gray	Brown	Violet-gray
27	Butyrospermol	VII	R = OH, Δ7,8, Δ24,25	0.22	Yellow	Violet	—
28	α-Euphorbol	X	R = OH, Δ8,9	0.24	Brown-gray	Red	Violet
29	Lanosterol	VIII	R = OH, Δ8,9, Δ24,25	0.33	Yellow	Brown	Violet
30	Cycloartenone	IXa		0.67	Yellow	Brown	Brown
31	Euphone	VIIa		0.73	Yellow	Yellow-brown	Brown
32	Butyrospermone	VII	R = C=O, Δ7,8, Δ24,25	0.78	Yellow	Brown	Gray
33	Agnosterol	VIII	R = OH, Δ6,7, Δ9,11, Δ24,25	0.88	Yellow	Yellow	Brown-yellow
34	Dihydrobutyrospermyl acetate	VII	R = OAc, Δ7,8	0.90	Gray	Red	Violet
35	Euphene	VIIa	$=CH_2$ instead of C=O	0.96	Brown	Gray-brown	Brown-yellow

value increases when the double bond is transposed from the 18, 19 (No. 5) *via* the 12, 13 (No. 6) to the 14, 15 position (No. 8). The R_F value for the saturated analogue (No. 9) is still higher. This appears to indicate that in the three unsaturated substances the double bonds become less polar or less important for adsorption in the sequence given.

In the tetracyclic series, (VII) has almost the same R_F value, whether the double bonds are in the 24, 25 and the 8, 9 positions (No. 24), in the 24, 25 and the 7, 8 positions (No. 27) or in the 24, 25 and the 9, 11 positions (No. 25).

It is somewhat surprising on the other hand that in the lanosterol series (VIII), three double bonds (No. 33) make the compound migrate more quickly than two (No. 25, 26).

Undoubtedly, a more complete study of this class of compounds will reveal the inherent regularities more clearly.

Experimental procedure

For the preparation of 5 glass plates (20 × 20 cm) a mixture of 50 g of alumina G (Merck) and 100 ml of distilled water was used. The well-shaken mixture was applied to a thickness of 0.25 mm with a Desaga apparatus. After 1 h at room temperature, the plates were dried for 30 min at 125° and kept in a desiccator.

The base line was fixed at a distance of 3 cm from the rim of the plate and the compounds were applied in chloroform solution by means of a micro-pipette. The distance between samples on the same plate was about 2 cm.

The development of the chromatogram with the above-mentioned mixture was carried out in one dimension, at 23°. Within 1 h, the solvent rose a distance of 12 cm. At the end of the development, the height to which the liquid rose was noted; after a further 10 min at room temperature, the plates were dried for 5 min at 120°.

The triterpenes were detected by spraying with three reagents: (A) antimony trichloride, 20% in chloroform, (B) antimony pentachloride, 20% in chloroform, (C) acetic anhydride (10%) and sulphuric acid (10%) in absolute alcohol.

After spraying, the plates were dried at 120° for 5 min.

Acknowledgements

The authors wish to thank Drs. J. McLEAN and W. LAWRIE of the Royal College of Science and Technology, Glasgow, for samples of the triterpenes.

Department of Organic Chemistry, Hebrew University, Jerusalem (Israel)

R. IKAN
J. KASHMAN
E. D. BERGMANN

1 R. TSCHESCHE, F. LAMBERT AND G. SNATZKE, *J. Chromatog.*, 5 (1961) 217.
2 R. TSCHESCHE AND A. K. SEN GUPTA, *Ber.*, 93 (1960) 1903.
3 A. F. THOMAS AND I. M. MULLER, *Experientia*, 16 (1960) 62.
4 S. HUNECK, *J. Chromatog.*, 7 (1962) 561.

Received August 27th, 1963

Emploi des complexes π en chromatographie sur couche mince

Dérivés polynitrés aromatiques et hydrocarbures aromatiques à noyaux condensés

De nombreux auteurs ont essayé d'améliorer les séparations chromatographiques en modifiant la composition de l'adsorbant utilisé. Dans ce but on imprègne en général l'adsorbant avec un produit susceptible de réagir sur les produits à séparer, par exemple en donnant réversiblement des complexes.

SONDHEIMER ET POLLAK ont appliqué avec succès ce principe à la résolution sur colonne de dérivés de l'acide cinnamique en imprégnant l'adsorbant avec un dérivé de la théophylline[1].

Dans le domaine de la chromatographie sur couche mince, on utilise également ce principe. C'est ainsi que l'on a eu recours à l'imprégnation avec du bisulfite de sodium (séparation des aldéhydes) ou avec de l'urée (séparation d'hydrocarbures linéaires d'hydrocarbures ramifiés[2]). HALMEKOSKI[3] utilise des molybdates et des tungstates qui forment des chelates avec les acides phénoliques. PASTUSKA[4] prépare les plaques de Kieselgel avec une solution d'acide borique pour améliorer la séparation des sucres. Lors de la séparation d'isomères éthyléniques, on a recours aux complexes avec le nitrate d'argent[5,6].

Ceci nous a amenés à généraliser l'utilisation des complexes moléculaires et plus particulièrement des complexes π en chromatographie sur couche mince.

Si deux catégories de produits, A et B, respectivement acide π et base π donnent réversiblement des complexes, on peut ou bien séparer les produits A en utilisant un produit B convenablement choisi comme complexant, ou bien faire l'inverse. Deux modes opératoires sont possibles: d'une part introduire le complexant dans la plaque par imprégnation préalable (technique habituelle de l'inversion de phase) ou d'autre part introduire le complexant dans le solvant. La solubilité du complexant dans le solvant utilisé oriente évidemment vers l'une ou l'autre de ces méthodes. Les complexes π étant généralement colorés, on peut suivre le fractionnement pendant la migration des produits et au besoin procéder à des développements multiples.

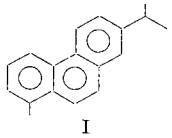
L'emploi d'un révélateur n'est pas indispensable, mais peut être utile pour augmenter la sensibilité. Nous avons choisi comme exemple le couple dérivés polynitrés aromatiques et hydrocarbures aromatiques à noyaux condensés.

Nous avons ainsi mis au point deux méthodes de séparation:

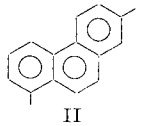
(a) Dérivés nitrés aromatiques courants grâce à un solvant saturé d'une base π (analyse des explosifs, critère de pureté).

(b) Hydrocarbures à noyaux condensés en utilisant des plaques imprégnées d'un acide π (applications possibles: huiles anthracéniques, hydrocarbures cancérigènes, structure de substances naturelles par aromatisation).

Dans le cas présent, l'intérêt de la méthode est particulièrement évident: sur plaques conventionnelles, les hydrocarbures polynitrés et surtout les hydrocarbures



I



II

à noyaux condensés se séparent mal, voire pas du tout. L'emploi des "plaques π" nous a notamment permis de séparer en un simple développement le rétène (I) du pimanthrène (II).

Cette technique nous a également permis de mettre en évidence des impuretés dans les produits commerciaux considérés comme purs (anthracène, biphényle, etc.).

Partie expérimentale

Dans les deux cas nous nous sommes servis de plaques de Kieselgel G selon Stahl (Merck) de format 14 cm × 10.5 cm, préparées manuellement, et activées 0.5 h à 110°. Les cuves sont d'un modèle courant sans "sursaturation".

(*a*) *Dérivés nitrés*

Les produits sont déposés en solution dans le chloroforme à raison de 1 % par constituant.

Solvant. Cyclohexane–chloroforme (50:50) saturé d'anthracène.

Mise en évidence. Les taches jaunes ou brunes sur fond blanc sont directement visibles. On augmente la sensibilité en examinant en lumière ultra-violette (3650Å).

Nous avons ainsi séparé en un simple développement les dérivés nitrés aromatiques suivants: *m*-dinitrobenzène (DNB), 2,4-dinitrotoluène (DNT), 2,4,6-trinitrotoluène (TNT), 2,4,6-trinitroanisole (TNA) et tétryl (Tet) (Fig. 1).

En l'absence de complexant, une séparation à peu près identique n'est obtenue qu'après trois développements successifs.

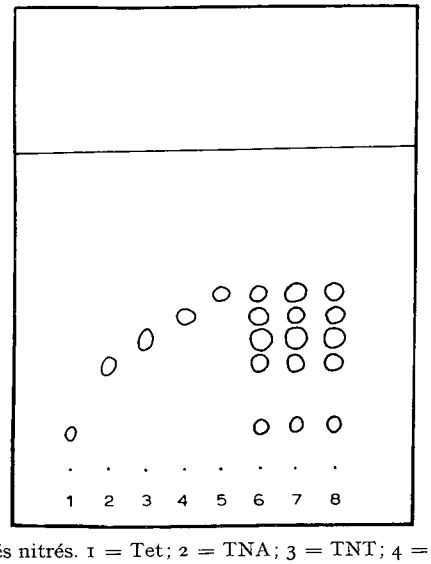


Fig. 1. Séparation de dérivés nitrés. 1 = Tet; 2 = TNA; 3 = TNT; 4 = DNB; 5 = DNT; 6,7,8 = mélange.

(*b*) *Hydrocarbures a noyaux condensés*

On commence par imprégner les plaques par ascension jusqu'au bord supérieur d'une solution benzénique de trinitrobenzène (TNB) à 3 %. On laisse sécher verticalement à l'air les plaques avant de les utiliser.

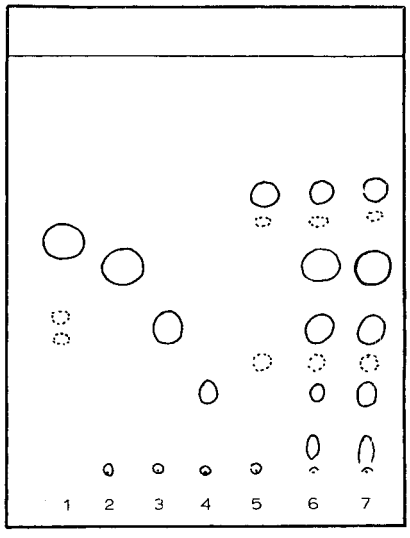


Fig. 2. Séparation d'hydrocarbures aromatiques. 1 = biphényle (2 impuretés); 2 = naphthalène; 3 = acénaphthène; 4 = anthracène; 5 = octahydroanthracène (2 impuretés); 6,7 = mélange et pyrène.

Solvant. Selon le R_F des hydrocarbures on se sert de cyclohexane pur ou avec 5 % d'acétate d'éthyle. Il est à noter que l'on peut aussi dans une certaine mesure faire varier globalement les R_F en changeant la concentration de TNB dans le solvant d'imprégnation.

Révélation. Les hydrocarbures sont visibles sous forme de taches jaunes ou brunes sur fond blanc. La meilleure révélation est obtenue par pulvérisation d'une

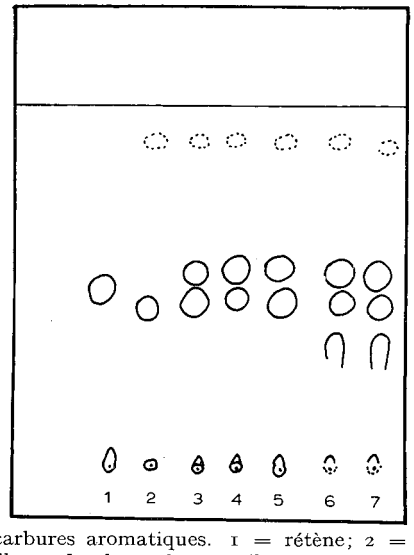


Fig. 3. Séparation d'hydrocarbures aromatiques. 1 = rétène; 2 = pimanthrène (1 impureté); 3,4,5 = mélange des deux; 6,7 = mélange des deux et chrysène.

solution de pentachlorure d'antimoine à 20 % dans le tétrachlorure de carbone, éventuellement suivie d'une deuxième pulvérisation d'acide sulfurique–paraformaldéhyde. Les taches sont alors très diversement colorées: orange à bleu-noir sur fond blanc.

Nous avons ainsi réussi à séparer les hydrocarbures suivants: pyrène, anthracène, acénaphtène, octahydroanthracène, naphtalène (solvant: cyclohexane) (Fig. 2), et sur une autre plaque le chrysène, le rétène et le pimanthrène (solvant: cyclohexane–5 % acétate d'éthyle) (Fig. 3). Les R_F s'échelonnent de 0.15–0.60 avec des taches très nettes alors que sans complexant les produits donnent des R_F allant de 0.45–0.60.

Il est évident que les modalités d'application du principe décrit ici sont très variées, tant en ce qui concerne la technique d'imprégnation de la plaque que pour la nature de l'agent complexant utilisé.

*Institut de Chime**,
Strasbourg (France)

M. FRANCK-NEUMANN
P. JÖSSANG

1 E. SONDHEIMER ET I. E. POLLAK, *J. Chromatog.*, 8 (1962) 413.
2 E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, Göttingen, Heidelberg, 1962, p. 40.
3 J. HALMEKOSKI, *Suomen Kemistilehti*, 35 (1962) 39.
4 G. PASTUSKA, *Anal. Chem.*, 179 (1961) 427.
5 B. DE VRIES, *Chem. Ind. (London)*, (1962) 1049.
6 L. J. MORRIS, *Chem. Ind. (London)*, (1962) 1238.

Reçu le 3 août 1963

* 2, Rue Goethe, Strasbourg.

Dünnschichtchromatographisches Verhalten herbicidwirksamer Verbindungen

Wir haben im Rahmen unserer analytischen Untersuchungen über Pesticide auch die Möglichkeiten des Nachweises, der Trennung und der Bestimmung herbicidwirksamer Verbindungen auf dünnschichtchromatographischem Wege geprüft. Die Arbeiten wurden vorerst mit *sym.*-Triazin-Derivaten und Phenoxyalkancarbonsäureester begonnen. Hierbei sollte vor allem die Leistungsfähigkeit der Methode gegenüber chemisch sehr ähnlichen Verbindungen untersucht werden.

Triazine

In die Arbeit wurden folgende *sym.*-Triazine einbezogen: 2-Methoxy-4-äthylamino-6-isopropylamino- (Atraton), 2-Methoxy-4,6-bis-(isopropylamino)- (Prometon), 2-Methylmercapto-4,6-bis-(isopropylamino)- (Prometryn), 2-Chlor-4,6-bis-(äthylamino)- (Simazin), 2-Chlor-4,6-bis-(isopropylamino)- (Propazin) und 2-Chlor-4-äthylamino-6-isopropylamino- (Atrazin).

Als geeignetes Sprühreagenz für die O- und S-haltigen Verbindungen (Prometon, Atraton, Prometryn) erwies sich Dragendorffs-Reagenz nach MUNIER UND

MACHEBOEUF[1], das schon bei schwachem Besprühen der Platte orange-braune Flecken auf hellgelbem Grund hervorruft. Die Cl-haltigen Derivate werden erst nach dem Transparentsprühen als weisse Flecken sichtbar. Während mit diesem Reagenz für die O- und S-haltigen Verbindungen ausreichende Empfindlichkeiten erreicht werden, befriedigen diese bei den Cl-haltigen nicht. Eine bessere Empfindlichkeit für diese Gruppe ergibt sich bei Verwendung der Jodplateat-Lösung (modifiziert für Alkaloide und verschiedene Heterocyclen[1]), mit der man nach dem Besprühen bis zur Transparenz weisse Flecken auf kräftig rosafarbenem Untergrund erhält.

Liegen alle 6 Verbindungen im Gemisch vor, so hat es sich als zweckmässig erwiesen, die Platte zuerst schwach mit Dragendorffs Reagenz und dann mit Jodplateat bis zur Transparenz zu besprühen. Ferner war eine neutrale 0.25%ige $KMnO_4$-Lösung für alle sechs Triazine, besonders aber für Simazin recht brauchbar. Das $AgNO_3$–Phenoxyäthanol-Reagenz nach MITCHELL[2] kann ausser für Simazin und Atrazin[3] auch für Propazin und Prometryn benutzt werden. Nach unseren Erfahrungen eignet sich jedoch dieses Verfahren nur für Mengen über 5 μg; die untere Erfassungsgrenze konnte nicht genau festgelegt werden. Als wesentlich empfindlicher für die Cl-haltigen Triazin-Derivate zeigte sich dagegen die Sprühmethode mit 0.1 *N* $AgNO_3$ in 3 *N* HNO_3 (Nachbehandlung: 5 Min. bei 80°, 10–15 Stunden im Tageslicht). Die lange Entwicklungszeit, die auch durch U.V.-Bestrahlung nicht verkürzt werden kann, steht allerdings der Anwendung als Schnellmethode entgegen. In Tabelle I sind die unteren Erfassungsgrenzen zusammengestellt, die einwandfrei reproduziert werden konnten.

Eine befriedigende Trennung der sechs Triazin-Derivate wurde in einer Zweistufen-Entwicklung mit Chloroform–Diisopropyläther (3:2) erreicht (Fig. 1).

TABELLE I

UNTERE ERFASSUNGSGRENZEN (IN μg)

	Dragendorff	*Jodplateat*	*0.1 N $AgNO_3$–3 N HNO_3*	*0.25% $KMnO_4$*
Prometon	2,5	3.0	—	2.0
Atraton	2.0	10.0	—	2.0
Prometryn	1.0	2.0	—	1.0
Simazin	1.0	1.0	0.5	0.5
Atrazin	3.0	2.5	0.3	1.5
Propazin	1.5	1.0	0.1	1.5

Das in Pflanzenschutzmitteln zusammen mit Triazinen, z.B. neben Simazin angewendete 3-Amino-1,2,4-triazol kann ebenfalls mit Jodplateat sichtbar gemacht werden (Erfassungsgrenze: 0.25 μg). Es lässt sich im System Essigester–Methanol (4:1) von Simazin trennen. Beide Produkte können so z.B. in Handelspräparaten nachgewiesen werden.

Phenoxyalkancarbonsäureester

Von den "Phenoxyalkancarbonsäureestern" wurden folgende Derivate untersucht: 2-Methyl-4-chlorphenoxyessigsäure-hexylester (MCPA-hexyl) und -(2-butoxyäthyl)-ester (MCPA-butoxyäthyl), α-(2-Methyl-4-chlorphenoxy)-propionsäure-hexylester (MCPP-hexyl), -äthylester (MCPP-äthyl), -(2-butoxyäthyl)-ester (MCPP-butoxyäthyl)

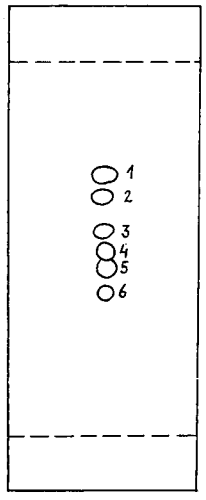


Fig. 1.

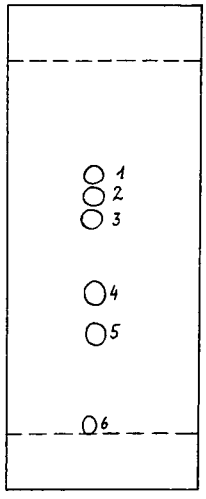


Fig. 2.

Fig. 1. 1 = Prometryn; 2 = Propazin; 3 = Atrazin; 4 = Prometon; 5 = Simazin; 6 = Atraton. System Chloroform–Diisopropyläther (3:2), zwei Stufen.

Fig. 2. 1 = MCPP-hexyl; 2 = MCPA-hexyl; 3 = MCPP-äthyl; 4 = MCPP-butoxyäthyl; 5 = MCPA-butoxyäthyl; 6 = MCPP-hydroxybutyl. System Cyclohexan–Diisopropyläther (5:1), zwei Stufen.

und -(3-hydroxybutyl)-ester (MCPP-hydroxybutyl). Eine Trennung der sechs Verbindungen ist mit Cyclohexan–Diisopropyläther (5:1) bei zweistufiger Arbeitsweise im gleichen System möglich (Fig. 2). Im Gegensatz zur Triazingruppe verhalten sich die Ester einheitlich gegenüber den Detektionsmitteln. Die Flecken sind nach Besprühen mit Rhodamin B (0.5 % in Äthanol) im U.V.-Licht sichtbar; die Erfassungsgrenze (20 μg) ist jedoch völlig unbefriedigend. Sie kann aber durch Besprühen mit $SbCl_5$ in CCl_4 (1:4) bis zur Transparenz und Nachbehandlung bei 105° auf 0.5 μg pro Komponente gesenkt werden. Es entstehen braune bis violette Flecke.

Für alle beschriebenen Arbeiten wurden handgegossene Platten, 100 × 200 mm, mit 2 g Kieselgel G (Merck, nachgesiebt) pro Platte, luftgetrocknet, verwendet. Laufstrecke: 10 cm. Die manuelle Herstellung war bei dieser und geringerer Plattengrösse (50 × 100 mm) notwendig, um gleichmässig ausgebildete Schichten mit reproduzierbarer Dicke zu erhalten. Die mit dem Desaga-Streichgerät (nach Stahl) hergestellten Schichten erfüllten diese Bedingungen nicht.

Biologische Bundesanstalt für Land- und Forstwirtschaft,
Institut für Pflanzenschutzmittelforschung,
Berlin-Dahlem (Deutschland)

HANNS G. HENKEL
WINFRIED EBING

[1] *Anfärbereagenzien für Dünnschicht- und Papier-Chromatographie*, Merck A.G., Darmstadt.
[2] L. C. MITCHELL, *J. Assoc. Offic. Agr. Chemists*, 44 (1961) 643.
[3] A. MAJOR, *J. Assoc. Offic. Agr. Chemists*, 45 (1962) 679.

Eingegangen den 26. August 1963

Die Herstellung einer einfachen Deckplatte die die Färbung von Dünnschichtchromatogrammen mit flüchtigen Reagenslösungsmitteln bei höheren Temperaturen ermöglicht

Verschiedene chemische Reaktionen mit Hilfe welcher man nach Dünnschichtchromatographie die getrennten Substanzen sichtbar macht, zum Beispiel beim Nachweis von Zucker[1], Aminosäuren[2], Glykolipiden[3], usw., erfordern höhere Temperaturen und bestimmte Detektionszeiten. Das Problem der Anfärbung bei höheren Temperaturen besteht darin, dass nach der Aufsprühung des Nachweisreagenses das Lösungsmittel sich viel zu schnell verflüchtigt, was zur Folge hat, dass das eigentliche Nachweisreagens viel zu wenig mit den getrennten Substanzen reagiert, sie nicht anfärbt, oder wie in den obengenannten Fällen[1–3] die ganze Dünnschichtoberfläche anfärbt und hiermit die Identifizierung der Substanzen erschwert.

Um die Verflüchtigung der Lösungsmittel während der Anfärbung bei höheren Temperaturen zu unterbinden, verfertigten wir eine Deckplatte an, die der Forderung auf stabile Zusammensetzung des Sprühreagenses auch bei höheren Temperaturen Rechnung trägt.

Durchführung der Detektion

Nach beendeter Chromatographie wird das Adsorptionsmaterial von den Plattenrändern in einer Breite von 1.5 cm (im oberen Teil der Trägerplatte 2.5 cm) durch Abschaben entfernt (Fig. 1, rechtes Bild) das Chromatogramm mit der Reagenslösung besprüht, mit der Deckplatte zugedeckt (Fig. 2) und im Trockenschrank erhitzt. Nach bestimmter Zeit (bei Aminosäuren mit Ninhydrinreagenslösung 10 Min. bei 105°), wird die Deckplatte abgenommen, das Dünnschichtchromatogramm im Trockenschrank getrocknet und hiermit die Anfärbung beendet. Mit dieser Art der Anfärbung erzielt man eine genaue Anfärbung der getrennten Substanzen, während das Adsorptionsmaterial (Kieselgel G) nicht, oder nur sehr wenig mitgefärbt wird.

Herstellung der Deckplatte

Auf eine Glasplatte von der selben Grösse wie die Trägerplatten (wir benutzten Platten 20 × 20 cm) werden nach Berauhung der Plattenrändern mit Glaspapier 1 cm breite Glasstreifen mit Wasserglaslösung angeklebt (Fig. 1, linkes Bild). Der obere Glasstreifen wird nicht am Plattenrande sondern um 1 cm weiter zur Mitte angeklebt um im Falle eines Ansaugens der Deckplatte an die Trägerplatte während des Färbens Raum zur Hebung der Deckplatte zu geben.

Zur bequemeren Handhabung wird auf die Oberseite der Deckplatte ein Glasstreifen (4 × 3 × 1 cm) mit seiner Längstkante angeklebt (Fig. 3, linkes Bild).

Im Falle das wir nur in einem Teile des Dünnschichtchromatogramms die Substanzen durch Anfärbung identifizieren wollten, während wir sie aus dem anderen Teile zur quantitativen Analyse isolierten, arbeiten wir mit schmäleren Deckplatten wie eine auf Fig. 4 dargestellt ist.

Obwohl wir mit den obenbeschriebenen Deckplatten gute Erfolge erzielten entwickelten wir in letzter Zeit eine neue Deckplatte, die grösser als die Trägerplatte ist und deren angeklebte Glasstreifen um 1 mm höher sind als die Trägerplatte mit der

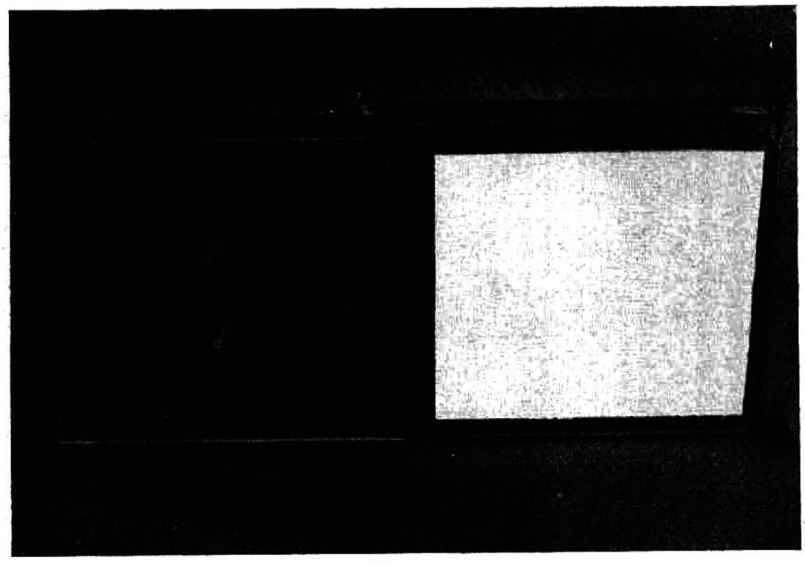


Fig. 1.

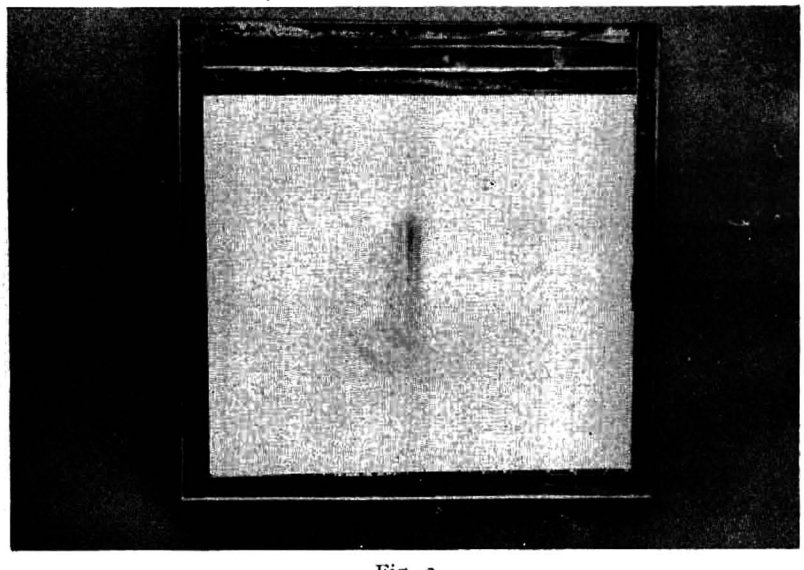


Fig. 2.

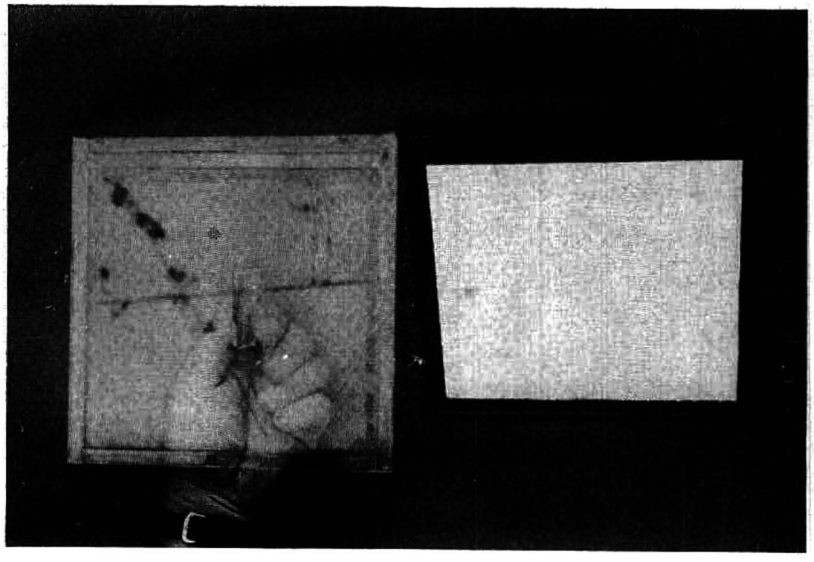


Fig. 3.

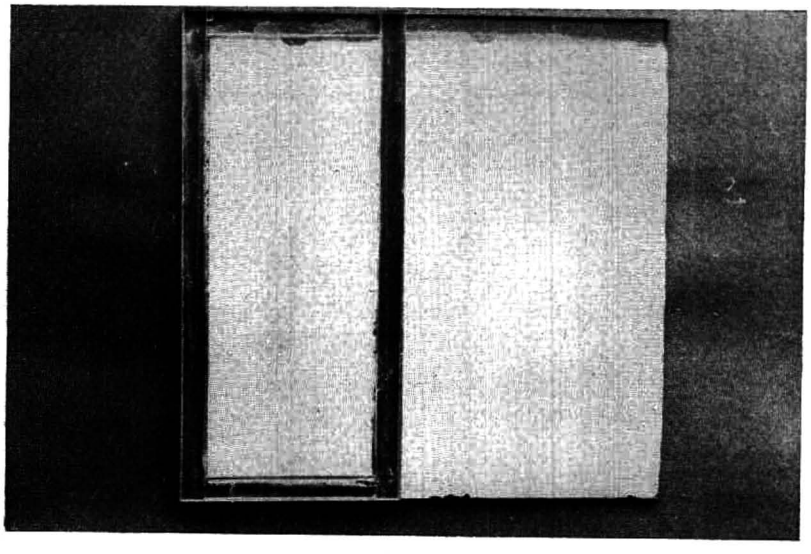


Fig. 4.

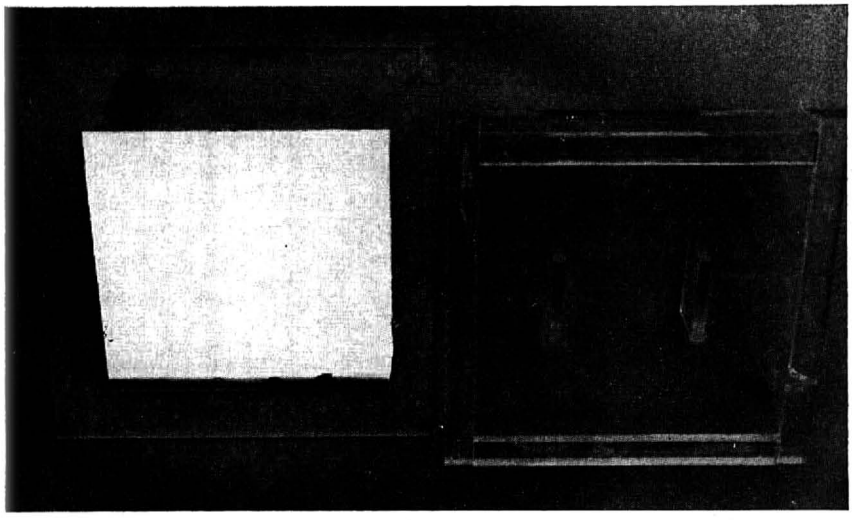


Fig. 5.

Adsorptionsschicht zusammen. Die Anfärbung erfolgt dann so, dass die Trägerplatte auf eine grössere Glasplatte gelegt, und mit der Deckplatte eingedeckt wird (Fig. 5). Die untere Glasplatte mit der Deckplatte ergibt in diesem Falle eine Reaktionskammer. Diese neue Methode der Anfärbung bringt den Vorteil dass das Adsorptionsmaterial von den Plattenrändern der Trägerplatte nicht abgeschabt werden muss und die ganze Platte zur Chromatographie ausgenützt werden kann.

Lehrstuhl für Analytische Chemie, Chemische Fakultät der Slowakischen Technischen Hochschule, Bratislava (Tschechoslowakei) L. PORGESOVÁ

Lehrstuhl für Medizinische Chemie, Komensky-Universität, Bratislava (Tschechoslowakei) E. PORGES

[1] E. D. MOFFAT UND R. J. LYTLE, *Anal. Chem.*, 31 (1959) 926.
[2] G. PASTUSKA, *Z. Anal. Chem.*, 179 (1961) 427.
[3] H. WAGNER, L. HÖRHAMMER UND P. WOLFF, *Biochem. Z.*, 334 (1961) 175.

Eingegangen den 28. August 1963

Thin-layer chromatography of carboxylic acids and keto acids of biological interest*

In conducting some biological investigations with which our institute is concerned[1] we had to tackle the problem of separating and identifying some compounds (amino acids, acids and sugars) present as mixtures in the water–alcohol extracts of plant cells (Chlorella).

* Work supported by a grant from the National Research Council of Italy.

Paper and ion-exchange chromatography gave satisfactory results with amino acids, but did not prove as efficient and easy to carry out with organic acids, particularly keto acids, which are of primary importance as amino acid precursors.

The recent extension of thin-layer chromatography to water-soluble substances led us, in view of the favourable results already reported[2,3], to try to develop a separation technique for the most important acids and keto acids of biological interest. The results we obtained can be regarded as promising.

Experimental

Suitably sized ground-glass plates washed with acetone were used as supports. The adsorbent layer consisted of silica gel (Kieselgel Merck, also containing $CaSO_4$ as binder) washed with HCl (1:1), then with water and finally with a 0.1 % w/v aqueous solution of EDTA. This treatment removed from the adsorbent solid iron and other interfering ions. As the $CaSO_4$ was also removed, it was replaced by adding 13 g of $CaSO_4 \cdot {}^1/_2H_2O$ per 100 g of silica gel. After stirring, the mixture was suspended in water (10:35) and poured on the plate (10 mg/cm^2 of surface). The layer was made homogeneous by means of an electromagnetic vibrator and then dried for about 45 min in an oven at 105°.

Development

The solvents that gave the most satisfactory results for the separation of the compounds and the compactness of the spots were (A) propanol–28° Bé ammonia (70:30) and (B) ethanol–chloroform–28° Bé aq. ammonia–H_2O (70:40:20:2).

After the solvent had travelled 13 cm from the starting line, the chromatoplate was dried by means of an infrared lamp, allowed to cool and finally sprayed with a 0.1 % solution of 2,6-dichlorophenolindophenol in 95 % alcohol. A subsequent short

TABLE I

R_F VALUES FOR CARBOXYLIC AND KETO ACIDS

Solvent A: Propanol–28° Bé ammonia (70:30)
Solvent B: Ethanol–$CHCl_3$–28° Bé ammonia–H_2O (70:40:20:2)

Acid	$R_F \times 100$ *in*	
	Solvent A	*Solvent B*
Adipic	27	16
Lactic	42	35
Succinic	17	9
Oxalic	4	0
Malic	7	4
Glycolic	30	22
Fumaric	20	12
α-Ketoglutaric	17	10
β-Ketobutyric	53	45
Pyruvic	5	3
Dehydroascorbic	55	46
Ascorbic	22	14
Levulinic	50	52

heating brought up all acidic compounds as pink spots on a sky-blue background. If the heating was prolonged and the chromatoplate carefully brought near to the infrared lamp, the pink spots given by keto acids turned white, whereas those given by carboxylic acids remained pink. In this way it was possible to distinguish keto acids from other acids. Exposure of the plate to ammonia vapours for a few seconds resulted in a sharper definition of the pink spots.

Results

Adipic, succinic, glycolic, fumaric, lactic, malic, oxalic, ascorbic, dehydroascorbic, β-ketobutyric, α-ketoglutaric, levulinic and pyruvic acids were subjected to the chromatographic procedure described above.

In Table I are listed the R_F values in the two solvents used.

Experiments are being conducted with the object of extending to other compounds, including sugars, the technique developed in the present work.

Institute of Agricultural Chemistry,
University of Padua (Italy)

C. PASSERA
A. PEDROTTI
G. FERRARI

[1] G. FERRARI, C. PASSERA AND R. CULTRERA, *Ric. Sci.*, 3 (1963) 181.
[2] D. BRAUN AND H. GEENEN, *J. Chromatog.*, 7 (1962) 56.
[3] H. J. PETROWITZ AND G. PASTUSKA, *J. Chromatog.*, 7 (1962) 128.

Received September 11th, 1963

J. Chromatog., 14 (1964) 289–291

Ion flotation of inorganic ions

1. The concentration of uranium

Surface active agents may be used for separation processes in a variety of ways such as adsorbing them on solid supports[1], in liquid phases or on a gas–liquid interface[2].

The possibilities of the last-mentioned, namely ion flotation or froth flotation, have been outlined by several workers[3–6]. As in ion exchange processes, however, the adsorption of ions on the surface of the air bubbles formed from an aqueous solution of surface active agents will depend on the ionic state of the ion to be adsorbed, the structure and distribution of the surface active agent (not only on its functional groups) and the pH and ions present in the solution. Thus exact conditions for optimum concentration have to be worked out for each particular case.

In this preliminary communication we wish to describe a typical case, the concentration of UO_2^{++} ions from a solution of ammonium carbonate.

A number of cationic surface active agents was compared in a preliminary study using the apparatus shown in Fig. 1. Optimum results were obtained with benzethonium chloride. The effects of the carbonate concentration, of the concentration of the surface active agent and of the uranyl ion concentration are shown in Figs. 2 and 3.

The first and most concentrated foam fractions were analysed for their surface active agent and uranyl ion contents and gave ratios of 4:1, indicating the formation of a salt of the type $R^{+}_{4}\ UO_2(CO_3)_3^{4-}$ in this process. For the determination of

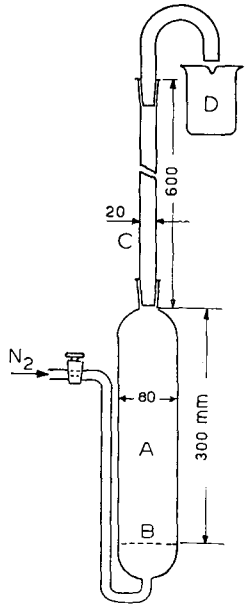


Fig. 1. Apparatus for foam extraction. A = Foam chamber, B = G2 porous septum; C = Glass column; D = Foam container.

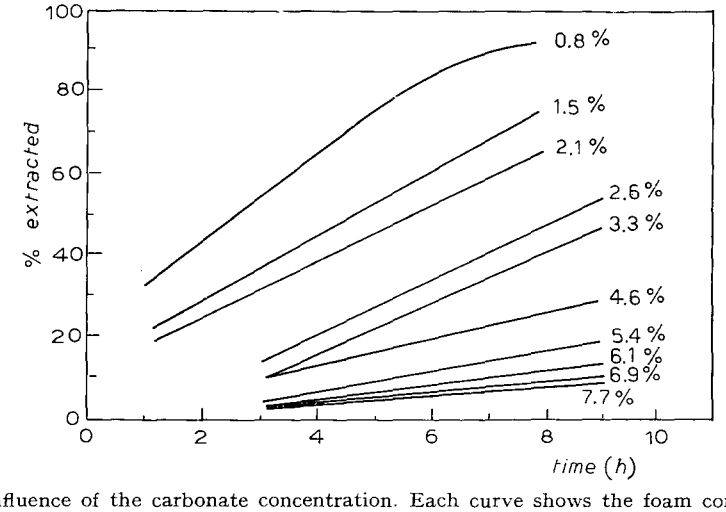


Fig. 2. Influence of the carbonate concentration. Each curve shows the foam concentration of uranyl ion with a flow rate of 75 ml of N_2/min and 0.2% of BZT solution and an initial concentration of 0.01% $UO_2(NO_3)_2 \cdot 6H_2O$. Each curve represents a concentration of ammonium carbonate (in g/100 ml).

benzethonium, spectrophotometry at 240 mμ gave good results in the required range of concentrations. Experiments with anionic exchange resins showed similar adsorptions with various carbonate concentrations as obtained in foams. It seems that a

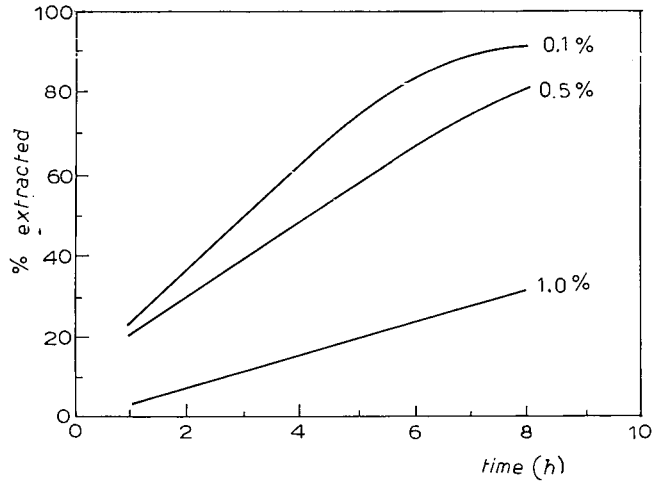


Fig. 3. Effect of foaming agent concentration. The foam concentration of UO_2^{++} with a flow rate of 75 ml of N_2/min and 3.75% ammonium carbonate and an initial concentration of 0.01% $UO_2(NO_3)_2 \cdot 6H_2O$. The curves show the % extracted with time for 0.1%, 0.5% and 1% of BZT.

broad analogy between ion flotation and ion exchange can be inferred even for the working conditions such as carbonate concentration, pH, etc. A similar correlation was also noted in froth flotation of the EDTA-complex of U(VI) which may be separated at pH 8 (where it is anionic) and not at pH 3 where it is cationic.

Paper chromatographic experiments as outlined in a previous communication[7] yielded the same information as actual flotation experiments. A mixture of the surface active agent and uranyl ions remained strongly adsorbed near the point of application when developed with 1% ammonium carbonate solution but was separated into a fast moving uranium spot and an adsorbed surface active agent spot when developed with 10% ammonium carbonate.

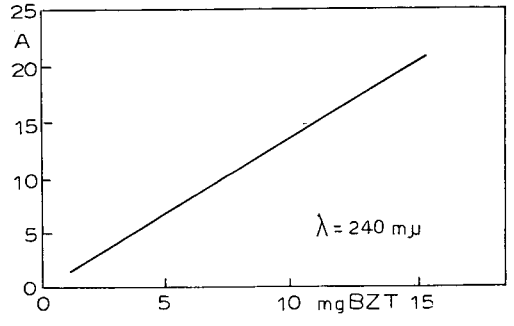


Fig. 4. Absorption at 240 mμ plotted against concentration of BZT (in mg).

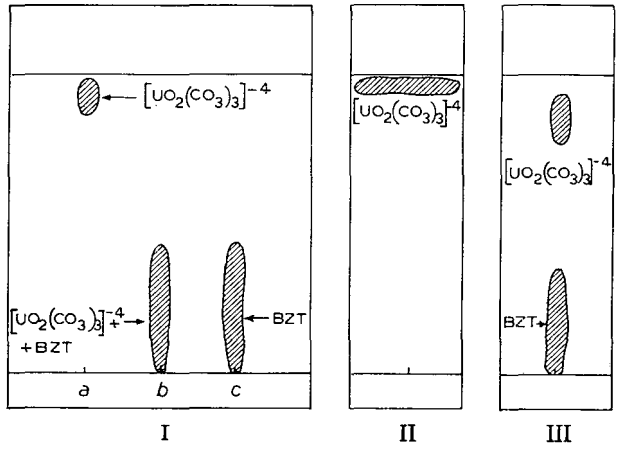


Fig. 5. Paper chromatograms of mixtures of uranyl carbonate and BZT. Paper: Whatman No. 1. I: a–c solvent = 1% aqueous ammonia carbonate; a = uranium carbonate only; b = mixture of uranium carbonate and BZT; c = BZT only. II: solvent = 1% aqueous ammonium carbonate containing 3% BZT. A spot of uranium carbonate was placed on the paper. III: solvent = 10% aqueous ammonium carbonate. A mixture of uranyl carbonate and BZT was chromatographed and separated into a fast moving uranium spot and a strongly adsorbed BZT spot.

The details of this work as well as some other separations of radiochemical interest will be described elsewhere.

Laboratorio di Chimica delle Radiazioni del C.N.E.N. e Istituto Superiore di Sanità, Centro di Chimica Generale del C.N.R., Istituto di Chimica Generale ed Inorganica dell'Università di Roma, Rome (Italy)

ALBERTO BAROCAS
CARLA IACOBELLI-TURI
FRANCO SALVETTI

1 D. E. WEISS, *Discussions Faraday Soc.*, 7 (1949) 142.
2 C. TESTA, *J. Chromatog.*, 5 (1961) 236; E. CERRAI AND C. TESTA, *J. Chromatog.*, 5 (1961) 442; 6 (1961) 443; 7 (1962) 112.
3 E. SCHÖNFELD AND R. SANFORD, *U. S. At. Energy Comm.*, NYO-9577 (1960).
4 F. SEBBA, *Nature*, 188 (1960) 736.
5 C. WALLING AND E. E. RUFF, *J. Phys. Chem.*, 61 (1957) 486.
6 E. RUBIN AND E. GADEN, *New Chemical Engineering Separation Techniques*, Interscience, New York-London, 1962, p. 319.
7 E. FARULLA, C. IACOBELLI-TURI, M. LEDERER AND F. SALVETTI, *J. Chromatog.*, 12 (1963) 255.

Received September 5th, 1963

A modified method for the separation of basic amino acids on the automatic amino acid analyzer*

The quantitative analysis of amino acids in biological preparations has been greatly simplified by the automatic ion-exchange chromatographic method of SPACKMAN, STEIN AND MOORE[1]. Frequently, free amino acids are encountered in urine, tissue homogenates and serum which are not well resolved by the 6 h procedure using the 15 cm column. These amino acids are readily separated and identified on the 50 cm column, but this method is time consuming (22 h) and uses large quantities of ninhydrin and eluting buffer.

KOMINZ[2] has reported an accelerated method for chromatography of basic free amino acids on the 50 cm column using a 0.70 *N* sodium citrate buffer pH 5.28. A system for analyzing basic amino acids in 80 min has also been developed by SPACKMAN[3]. Neither of these methods gives complete resolution of all the basic free amino acids which are found in urine, tissue homogenates and serum, and the second method requires several major modifications in the operation of the amino acid analyzer.

The present report describes a rapid method for analysis of basic amino acids which has been applied to serum, tissue homogenates, urine and protein hydrolysates. An analytical run takes $8^1/_3$ h and only requires the equipment and reagents regularly used with the Spinco model 120 amino acid analyzer. The method resolves 11 basic amino acids and the shortened operating time allows a 60 % saving in reagents.

A sample volume of 1–3 ml was applied to the 15 cm column which had been poured to a resin height of 21 cm with Spinco type 15A resin. The eluting buffer was the 0.37 *N* sodium citrate, pH 4.26, and was pumped through at 30 ml/h. The column

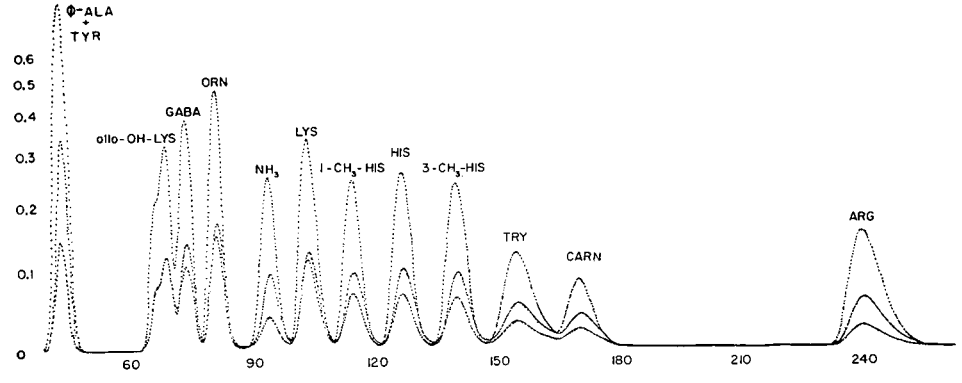


Fig. 1. Chromatogram of a synthetic amino acid mixture containing 0.5 μmole of each amino acid. The optical density is recorded on the ordinate and the effluent volume on the abscissa. The following abbreviations are used: ø-ala, L-phenylalanine; Tyr, L-tyrosine; allo-OH-Lys, DL-allo-hydroxy-lysine; GABA, γ-aminobutyric acid; Orn, L-ornithine; NH_3, ammonia; Lys, L-lysine; 1-CH_3-His, L-1-methylhistidine; His, L-histidine; 3-CH_3-His, L-3-methylhistidine; Try, L-tryptophan; Carn, L-carnosine; Arg, L-arginine.

* This work was supported by United States Public Health Service Research Grant No. AI-04152. Dr. KIRKPATRICK is a special post-doctoral fellow of the United States Public Health Service.

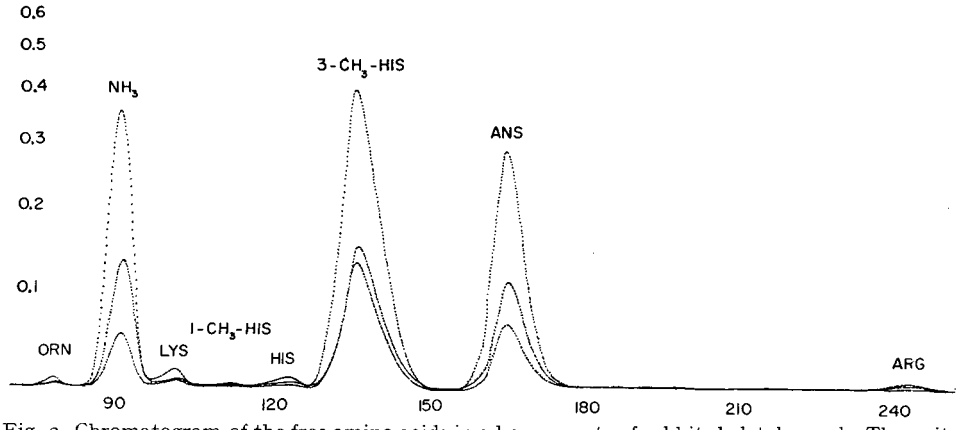


Fig. 2. Chromatogram of the free amino acids in a homogenate of rabbit skeletal muscle. The units are the same as Fig. 1. Ans represents L-anserine.

temperature at the beginning of elution was 30° and was increased to 50° at 4 h. Ninhydrin was added at 30 min at 15 ml/h.

The ability of this method to resolve 11 ninhydrin-reactive compounds in a synthetic mixture is illustrated in Fig. 1. The resolution was nearly equal to that obtained with the 50 cm column and the sharp, narrow peaks allowed accurate identification and quantitation. Fig. 2 shows the analysis of the free amino acids in a homogenate of rabbit skeletal muscle. The location of the peaks of 15 ninhydrin-reactive compounds is summarized in Table I.

TABLE I

LOCATIONS OF NINHYDRIN REACTIVE PEAKS

*Compound**	*Effluent to peak in ml*
L-Tyrosine, L-phenylalanine	44
D-Galactosamine	50 (shoulder on tyr-phe)
DL-Allo-δ-hydroxylysine**	68
γ-Aminobutyric acid	73
L-Ornithine	80
Ammonia	92
L-Lysine	102
L-1-Methylhistidine	114
L-Histidine	125
L-3-Methylhistidine	138
L-Tryptophan	158
L-Anserine	165
L-Carnosine	170
Creatinine	178
L-Arginine	241

* Tyrosine, phenylalanine, ammonia, lysine, histidine and arginine were contained in Spinco Type 1 amino acid calibration mixture, Lot No. CM 108. The other compounds were purchased from the California Corporation for Biochemical Research, Los Angeles 63, California.

** The shoulder on the ascending limb of the curve is believed to represent an impurity in the reagent standard.

This method of chromatographic analysis of basic amino acids has been used in this laboratory for several months and has allowed substantial savings in both time and reagents.

Department of Medicine,
University of Colorado Medical Center,
Denver, Colo. (U.S.A.)

CHARLES H. KIRKPATRICK
ROBERTA A. ANDERSON

[1] D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
[2] D. R. KOMINZ, *J. Chromatog.*, 9 (1962) 253.
[3] D. H. SPACKMAN, *Federation Proc.*, 22 (1963) 244.

Received August 16th, 1963

J. Chromatog., 14 (1964) 295–297

Chromatographic separation of histamine and some metabolites on cellulose phosphate paper*

The metabolism of histamine (M) in animals has been reviewed recently[1]. The main products of metabolism are 1-methyl-4-(β-aminoethyl)-imidazole (MeM), imidazole-4(5)-acetic acid (ImAA), imidazoleacetic acid riboside (ImAA-riboside), 1-methyl-imidazole-4-acetic acid (MeImAA), and acetyl-histamine (AcM). Quantitative analyses of ^{14}C-labelled histamine metabolites have been performed by crystallization of the different products as their respective picrates or *p*-iodophenyl-sulfonyl (pipsyl) salts[2,3]. These methods, however, are quite cumbersome and time-consuming.

Paper chromatography has been used by SCHAYER AND KARJALA[2] to separate radioactive metabolites of histamine. Three radioactive peaks were found using a solvent system composed of 1-butanol–ethanol–ammonia (80:10:30, v/v). The first peak contained ImAA-riboside, the second MeImAA and ImAA, and the third M, MeM and AcM. Good separation of histamine, histidine and acetyl-histamine can be obtained using Whatman No. 1 filter paper with isopropanol–0.25 *N* NH_4OH (3:1)[4]. In this system MeM can not be separated from M nor ImAA from MeImAA[5]. Thus, until now, it has not been possible to separate all of the above mentioned metabolites of histamine by paper chromatography.

In a preliminary effort to separate the different known metabolites of histamine by chromatography, a variety of papers were tried, Schleicher and Schüll papers No. 5984D, 2040A, blue R589D, white R589, 2043A, 2041, red R589, 598, 2043-D, 602 extra dense, 576, green R589, black R589D, 470 and 470A. Also, different solvents (as recommended by BLOCK *et al.*[6]) and ionophoresis at different pH values were evaluated. None of these techniques proved successful. Amberlite ion exchange resin loaded papers (Sa-2, Wa-2, Sb-2 and Wb-2) and Whatman cellulose ion exchangers (P-20, CM-50, DE-20 and ET-20) were tried next. Best results were achieved with the cation exchanger, cellulose phosphate paper (P-20). These results are reported below.

* This work was partially supported by grant-in-aid GRS-63-9 from the National Institute of Health.

Experimental procedure

Ascending and descending chromatography with P-20 cellulose phosphate paper was used. To recycle the paper through its H^+ and Na^+ forms we washed it in a downward direction successively with (1) 0.1 *N* HCl, (2) water, (3) 0.1 *N* NaCl, (4) water; each step proceeded for 24 h. To prepare a bi-functional cation exchanger the paper was washed with a solvent containing 0.1 *N* KCl and 0.001 *N* KOH, for 24 h and then washed with water. The washed papers were airdried. The chromatograms were developed with 0.1 *M* phosphate buffer (pH 7.5) for 1.5 h by the ascending technique and 3 h by the descending technique. The solvent front for the former reached 21 cm and for the latter 46 cm. The papers were dried in air and dipped in *p*-nitrobenzene diazonium fluoborate (Eastman No. P7078, 1 g in 100 ml acetone), and then sprayed with 0.1 *N* KOH in ethanol[7]. The stabilized diazotizing reagent was used in place of the recommended diazotized sulfanilic acid followed by Na_2CO_3 (BLOCK *et al.*[6]) because the latter reagent did not react with either MeImAA or MeM.

Results and discussion

Using unwashed cellulose phosphate paper with phosphate buffer, pH 7.5 as solvent, two zones resulted on the paper, one basic, the other acid as visualized with a bromocresol green indicator spray. The basic zone extended from the origin to about R_F 0.3; the acid zone from 0.3 to the solvent front. These zones could also be observed after treatment with the diazotizing color reagent; the lower zone (R_F 0–0.3) was dark brown due to formation of nitrobenzene, while the upper zone was light yellow. The zone formation may be due to non-equilibrium conditions of the ion exchange process or due to the separation of the solvent into two phases during development, as has been observed by POLLARD *et al.*[8].

Formation of two phases, however, did not seem to affect the separation of the compounds. As a matter-of-fact, histidine always migrated to the line of phase separation, giving a slightly distorted spot (see D, Fig. 1). Prewashing of the paper with either the developing buffer, HCl followed by NaOH, or KCl and KOH did not eliminate the two phases on the paper. Using paper washed with KCl and KOH improved the chromatography and facilitated better visualization of the MeImAA spot.

TABLE I

R_F VALUES OF SOME IMIDAZOLE DERIVATIVES* ON CELLULOSE PHOSPHATE

Imidazole derivatives	*Abbreviation*	R_F		*Color of spots (NBDFB reagent)***
		Ascending	*Descending*	
Histidine	D	0.29	0.32	Dark brown
Histamine	M	0.07	0.08	Dark brown
Methyl-histamine***	MeM	0.15	0.15	Yellow
Acetyl-histamine	AcM	0.46	0.48	Orange
Imidazoleacetic acid	ImAA	0.54	0.54	Reddish brown
Methyl-imidazoleacetic acid***	MeImAA	0.68	0.79	Orange

* Experimental details in text.

** 1 % (w/v) *p*-nitrobenzene diazonium fluoborate in acetone, followed by 0.1 *N* ethanolic KOH.

*** These compounds were obtained from Dr. R. W. SCHAYER, later purchased from Calbiochem, Los Angeles.

The color of the imidazole derivatives and their R_F values are given in Table I. A chromatogram of the imidazole derivatives is shown in Fig. 1, illustrating excellent separation of 6 compounds. To obtain even better separation of AcM and ImAA a chromatogram had to be run for 6 h, and the solvent was allowed to drip off the bottom of the paper.

ImAA-riboside could not be obtained for these studies, but its identity on paper probably could be determined by chromatography after acid hydrolysis (1 N HCl at 150°, for 6 h) as ImAA[9].

Although excellent separation of histamine derivatives is achieved, the method

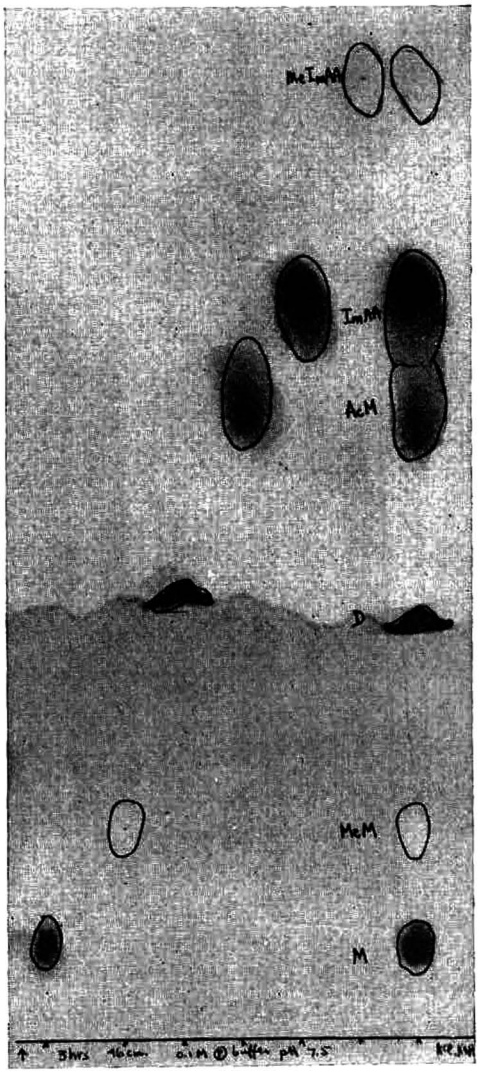


Fig. 1. Paper chromatogram of imidazole derivatives. P-20 cellulose phosphate paper; development time 3 h with 0.1 M phosphate buffer, pH 7.5, as solvent; descending development. D = histidine, all other abbreviations in text.

is not suitable for micro-amounts since the least detectable amount is *ca.* 50 μg for all investigated derivatives except for MeImAA for which *ca.* 100 μg is necessary. In contrast, only 1.0–10.0 μg quantities produce colored spots on regular cellulose paper.

Using the above technique, quantitative analysis of ^{14}C-histamine metabolites from embryonating chicken eggs is being investigated. Metabolic radioactive products are chromatographed by the described procedure with known compounds as markers, and the developed spots cut out and their radioactivity measured with a liquid scintillating spectrometer[10].

Departments of Avian Medicine and Agricultural Toxicology and Residue Research Laboratory, University of California, Davis, Calif. (U.S.A.)

MOSHE SHIFRINE
GUNTER ZWEIG*

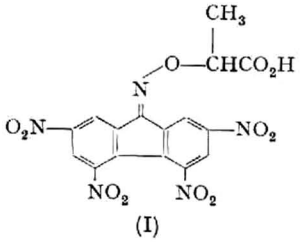
1 R. W. SCHAYER, *Physiolog. Rev.*, 39 (1959) 116.
2 R. W. SCHAYER, S. A. KARJALA, K. J. DAVIS AND R. L. SMILEY, *J. Biol. Chem.*, 221 (1956) 307.
3 R. W. SCHAYER AND J. A. D. COOPER, *J. Appl. Physiol.*, 9 (1956) 481.
4 M. SHIFRINE, L. E. OUSTERHOUT, C. R. GRAU AND R. H. VAUGHN, *Appl. Microbiol.*, 7 (1959) 45.
5 C. H. FISHEL, A. SZENTIVANYI AND D. W. TALMAGE, *J. Immunol.*, 89 (1962) 8.
6 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Chromatography and Paper Electrophoresis*, Academic Press, Inc., New York, 1958.
7 G. ZWEIG AND T. E. ARCHER, *J. Agr. Food Chem.*, 6 (1958) 910.
8 F. H. POLLARD, J. F. W. MCOMITE AND D. J. JONES, *J. Chem. Soc.*, (1955) 4337.
9 S. A. KARJALA, B. W. TURNQUEST (III) AND R. W. SCHAYER, *J. Biol. Chem.*, 219 (1956) 9.
10 E. A. DAVIDSON, (Packard Instruments Co.), *Tech. Bull.*, No. 4 (Rev. Jan. 1962) 1.

Received September 16th, 1963

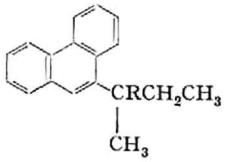
* Present address: Weizmann Institute of Science, Rehovoth, Israel.

Optical resolution of 9-*sec.*-butylphenanthrene by molecular complexation chromatography

KLEMM AND REED[1] described the use of columns of silicic acid impregnated with the optically active molecular complexing agent (+)- or (—)-α-(2,4,5,7-tetranitro-9-fluorenylideneaminoöxy)-propionic acid (I) for optical resolution of an ether and of an ester containing one center of asymmetry (on carbon) for each compound. In addition the method was used for optical resolution of a hydrocarbon wherein optical activity



(I)



(II): R=H
(III): R=OH

results from serious restriction to rotation around a pivotal bond of the biphenyl type. We now report the successful extension of the method to the partial optical resolution of 9-*sec.*-butylphenanthrene (II), a hydrocarbon containing a single center of asymmetry and expected to have a small specific rotation. The alkylarene II was selected for investigation because it contains the simplest alkyl group (devoid of isotopic labelling) possessing asymmetry when attached to an aryl moiety and because maximum overlap[2] of the angular fluorene and phenanthrene nuclei during molecular complexation should bring the centers of asymmetry in I and II into spatial proximity for maximal interaction.

Experimental

Synthesis of II. Methyl ethyl 9-phenanthryl carbinol (III, 47.1 g, m.p. 102–104°, 94% yield from 9-bromophenanthrene[3]) was dehydrated by distillation at 0.3 mm pressure. Treatment of the crude distillate with an equimolar amount of anhydrous picric acid in absolute ethanol gave 58 g (67 % yield from III) of yellow *9-(2-butenyl)-phenanthrene picrate*, m.p. 101–103°. *Anal.* Calcd. for $C_{18}H_{16} \cdot C_6H_3N_3O_7$: neut. equiv. 461.4. Found[4]: neut. equiv. 464. Chromatographic dissociation of the picrate using benzene and Alcoa F-20 alumina and distillation of the effluent gave slightly yellow *9-(2-butenyl)-phenanthrene*, 96% recovery, b.p. 137–142° (0.3 mm); infrared absorption bands at 905 cm^{-1} (strong, terminal methylene) and 825 cm^{-1} (medium, trisubstituted ethylene?); showing positive test with aqueous permanganate. *Anal.* Calcd. for $C_{18}H_{16}$: C, 93.06; H, 6.94. Found* (for sample of b.p. 140–141°): C, 92.74; H, 7.24.

Agitation of a mixture of 11.6 g of the preceding alkenylphenanthrene, 2 g of 5 % Pd–$BaSO_4$, and 75 ml of glacial acetic acid in hydrogen gas at 4 atm pressure for 5 h followed by removal of the catalyst and distillation gave 8.2 g (71%) of II, a faintly yellow liquid, b.p. 150–155° (0.3 mm); showing negative test with aqueous permanganate. *Anal.* Calcd. for $C_{18}H_{18}$: C, 92.26; H, 7.74. Found* (for sample of b.p. 151–152°): C, 92.38; H, 7.51.

II-picrate was obtained as yellow crystals from ethanol, m.p. 97–98.5°. *Anal.* Calcd. for $C_{18}H_{18} \cdot C_6H_3N_3O_7$: neut. equiv. 463.4. Found[4]: neut. equiv. 464.

Optical resolution. In a manner similar to that previously reported[1] a glass burette (1.5 cm in diameter) with a non-lubricated stopcock was packed (dry and without suction) with successive layers of glass wool, 2 cm of plain silicic acid, 50 cm of a mixture of 13 g of Johns-Manville Celite (anal. grade) and 27 g of silicic acid impregnated with (—)-I (8.9 % by wt.), and 2 cm of purified sand. The column was wrapped in aluminum foil. "Pure grade" cyclohexane (99 mole % minimum) was used as prewash for the column and as eluent for the 0.58 g of racemic II used. As soon as the red color of the molecular complex had reached the bottom of the column, fractions of 200 drops each were collected in tared test tubes at the average rate of 1 fraction per 26 h. Fractions were evaporated to constant weight in a stream of nitrogen, quantitatively diluted to 5 ml (the volume of the 1 dm polarimetric cell) with cyclohexane, and measured for optical rotation at 365 mμ and 24°[1] by means of a Perkin-Elmer model 141 polarimeter (accuracy of $\pm$0.002°). Only fractions 2–5 (of 11 total) showed significant rotations as follows (in order of appearance): 0.11 g, α = +0.008°, [α] = +0.36°; 0.17, +0.005°, +0.15°; 0.06, —0.005°, —0.42°; 0.05,

* Elemental analyses by Micro-Tech Laboratories, Skokie, Ill.

—0.007°, —0.70°. No significant rotations were found at wavelengths longer than 500 mμ.

Acknowledgement

This research was supported (in part) by the Directorate of Chemical Sciences, Air Force Office of Scientific Research under contract AF 49 (638)-473.

Department of Chemistry,
University of Oregon,
Eugene, Oreg. (U.S.A.)

L. H. KLEMM
K. B. DESAI
J. R. SPOONER, JR.*

[1] L. H. KLEMM AND D. REED, *J. Chromatog.*, 3 (1960) 364.
[2] L. H. KLEMM AND J. W. SPRAGUE, *J. Org. Chem.*, 19 (1954) 1464; J. LANDAUER AND H. MCCONNELL, *J. Am. Chem. Soc.*, 74 (1952) 1221.
[3] Y. S. ZAL'KIND AND K. V. BAL'YAN, *Zh. Obshch. Khim.*, 20 (1950) 1209; *C.A.*, 45 (1951) 1571.
[4] L. H. KLEMM, B. T. HO, C. D. LIND, B. I. MACGOWAN AND E. Y. K. MAK, *J. Org. Chem.*, 24 (1959) 949.

Received September 23rd, 1963

* NSF Undergraduate Research Participant.

Book Reviews

Thin-layer Chromatography, by JAMES M. BOBBIT, Reinhold Publishing Corporation, New York, 1963, xi + 208 pages, price $ 8.50.

This book gives a very detailed and complete account of the principles and techniques of thin-layer chromatography in the first 123 pages. Then follows a chapter on "specific applications of thin-layer chromatography" which consists of fifty odd pages of tables listing the compound type, the adsorbent, the developer, visualisation, "comments" and the reference number. In introducing the chapter the author states that specific R_F values are not given because of their notorious inconstancy. This remark would certainly be criticised by some authors. But even where R_F values are not reproducible it would help the reader to see exactly which compounds were separated and what differences in R_F values were obtained. These tables are thus only a very inadequate guide to the literature. The reference list contains 460 references, many as recent as 1963, and there is an adequate subject index.

Handbook of Analytical Chemistry, edited by L. MEITES, McGraw-Hill Book Company, Inc., New York, 1963, price £ 18.8.0.

This, to the reviewers' best knowledge, is the first chemical handbook to contain an entire section of 170 pages of data on techniques of separation, including 87 tables on chromatography.

For solvent extraction (by H. FREISER, *et al.*) there is a table classifying metal extraction systems and another summarising extraction procedures for all elements in alphabetical order. Adsorption chromatography (by H. STRAIN) is condensed to five pages, but this is admittedly difficult to present in tabular form. The paper chromatography of inorganic substances (by F. H. POLLARD AND G. NICKLESS) contains 21 tables giving the R_F values of metal ions and anions in numerous solvent systems, as well as some tables of paper chromatographic reagents. The paper chromatography of organic substances (by D. P. SCHWARTZ) gives a selection of R_F values of various compound classes in some of the more important solvent systems in over 20 tables, and some reagents in a three-page table. Evidently the right choice is rather difficult here considering the enormous amount of material published. There are 20 pages of retention volume tables in the section on gas chromatography (by R. L. PECSOK) covering a wide range of compounds. The section on "electrochromatography" (by A. KARLER) is somewhat more concerned with explanatory text than data and gives only a rather vague table of "typical applications", while from the previous sections some tables of M_g values would have been expected. Ion exchange (by H. F. WALTON) is again descriptive, summarising separations in a single table giving the conditions under which certain separations were carried out. This seems to the reviewer much less important than the tables of K_d values in which recent literature abounds, both for inorganic and organic substances. The editor and the authors of this handbook are to be congratulated on their success in compiling a set of tables from which, in spite of a certain lack of homogeneity, the analyst can find a method for a given problem or a lead on the possibilities of a given technique.

The remainder of the book (over 1000 pages) covers most analytical methods and their applications and there is a 66 page subject index arranged in such a way that it gives not only the page number but also the kind of method that is found thereon.

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

CALZOLARI, C., CERMA, E. AND BRUNI, G.: Application of chromatographic methods to the analysis of alimentary products. *Rass. Chim.*, 14 (1962) 182–194; *C.A.*, 58 (1963) 7293e — a review with 447 references.

3. TECHNIQUES I

DEYL, Z., PAVLÍČEK, M. AND ROSMUS, J.: (Paper chromatography in a centrifugal field). *Chem. Listy*, 57 (1963) 479–493 — a review with 53 references.

FINK, K., CLINE, R. E. AND FINK, M. R.: Paper chromatography of several classes of compounds: correlated R_F values in a variety of solvent systems. *Anal. Chem.*, 35 (1963) 389–398 — R_F values of 360 substances in 10 solvent systems.

HOUK, A. E. H.: Gravimetric evaluation of paper chromatograms. I. *J. Pharm. Sci.*, 52 (1963) 738–743.

KOUDELA, S.: (Chromatographic ruler). *Chem. Zvesti*, 17 (1963) 451–453.

PŘÍBELA, A.: (An apparatus for quantitative evaluation of paper chromatograms). *Chem. Zvesti*, 17 (1963) 689–692.

PŘÍBELA, A.: (Comparison of photometric evaluation of chromatograms in reflected and transmitted light). *Chem. Zvesti*, 17 (1963) 816–822.

Shandon Scientific Co. Ltd., (inventors: SHANDON, E. R. AND POWELL, R. H.): Improvements in paper-chromatography apparatus. *Brit. Pat.*, 929,578 (July 5, 1960); *Anal. Abstr.*, 10 (1963) 4982.

WONG, F. F. AND CARSON, J. F.: Drying manifold for spot tests and chromatograms on paper. *Chemist-Analyst*, 52 (1963) 20; *Anal. Abstr.*, 10 (1963) 4983.

7. PHENOLS

ANWAR, M. H.: Paper chromatography of monohydroxyphenols in vanilla extracts. *Anal. Chem.*, 35 (1963) 1974–1976.

TURUNEN, J.: (A paper-chromatographic method for the analysis of guaiacol derivatives). *Suomen Kemistilehti*, 35B (1962) 235–237; *C.A.*, 58 (1963) 6194g.

YUDINTSKAYA, A. I.: (R_F values of phenylazo derivatives of simple phenols). *Zh. Prikl. Khim.*, 34 (1961) 395; from *J. Chromatog., Chromatog. Data*, 10 (1963) D3 — R_F values of 18 substances.

ZWIMPFER, G. AND BÜCHI, J.: Die papierchromatographische Prüfung der Filix-Phloroglucide und der Rohfilicine. *Pharm. Acta Helv.*, 38 (1963) 663–675.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

PEGG, G. F.: R_F values of fusaric, alternaric and gibberellic acids. *Ann. Botany (London)*, 26 (1962) 219; from *J. Chromatog., Chromatog. Data*, 11 (1963) D16.

9. OXO COMPOUNDS

BARBER, E. D. AND LODGE, JR., J. P.: Paper chromatographic identification of carbonyl compounds as their 2,4-dinitrophenylhydrazones in automobile exhaust. *Anal. Chem.*, 35 (1963) 348–350.

DOKUNIKHIN, N. S. AND KOLOKOLOV, B. N.: (Effect of the position of halogen on behavior of monohaloanthraquinones in paper chromatography). *Zh. Vses. Khim. Obshchestva im. D.I. Mendeleeva*, 7 (1962) 597; *C.A.*, 58 (1963) 6759c.

LINKO, YU-YEN, MILLER, B. S. AND JOHNSON, J. A.: Quantitative determination of certain carbonyl compounds in pre-ferments. *Cereal Chem.*, 39 (1962) 263–272; *Anal. Abstr.*, 10 (1963) 1187.

10. CARBOHYDRATES

POMERANZ, Y., ROBINSON, R. J. AND SHELLENBERGER, J. A.: Evaluation of β-galactosidase (lactase) activity by paper chromatography. *Enzymologia*, 25 (1963) 157–166; *Anal. Abstr.*, 10 (1963) 4900.

STEPANENKO, B. N. AND SHCHERBUKHINA, N. K.: (On the chemical nature of the so-called "salep-mannana"). *Dokl. Akad. Nauk SSSR*, 151 (1963) 967–970.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BUSWELL, A. M., GILCREAS, F. W. AND MORGAN, G. B.: Paper chromatography for volatilic acid determinations. *J. Water Pollution Control Federation*, 34 (1962) 307–308; *C.A.*, 58 (1963) 6562h.

CHOREMIS, K. B., CONSTANTSAS, N. AND DANELATOS-ATHANASSIADIS, K.: Evidence for deviation in the metabolism of tyrosine in rheumatic fever. Preliminary communication. *Clin. Chim. Acta*, 8 (1963) 814–821.

CORSE, J., SONDHEIMER, E. AND LUNDIN, R.: 3-Feruoylquinic acid. A 3'-methyl ether of chlorogenic acid. *Tetrahedron*, 18 (1962) 1207–1210; *C.A.*, 58 (1963) 6738a.

HASHMI, M. H., ALI, E. AND UMAR, M.: R_F values of bromo-derivatives of some higher unsaturated fatty acids. *Pakistan J. Sci. Res.*, 14 (1962) 103; from *J. Chromatog., Chromatog. Data*, 10 (1963) D18.

HRUŠKA, V. AND DUCHOŇ, J.: (3-Methoxy-4-hydroxymandelic acid in the diagnosis of phaeochromocytomes). *Casopis Lekaru Ceskych*, 102 (1963) 962–966.

KOBAYASHI, T. AND MIYAZAKI, S.: Separation and determination of aliphatic dicarboxylic acids. III. Determination of acids separated on chromatographic paper. *Rept. Gov. Chem. Ind. Res. Inst. (Tokyo)*, 57 (1962) 147–151; *Anal. Abstr.*, 10 (1963) 4229.

KOMANOVÁ, E. AND KOMAN, V.: (Chromatographic determination of fatty acid content in edible oil). *Prumysl Potravin*, 13 (1962) 388–389; *Anal. Abstr.*, 10 (1963) 1216.

PAPKE, E. AND POHLOUBEK-FABINI, R.: Versuch zum papierchromatographischen Nachweis von α-Ketocarbonsäuren in Form einiger Thiosemicarbazone. *Pharmazie*, 18 (1963) 395–401 — extensive tables of derivatives of α-keto acids.

POHLOUBEK-FABINI, R. AND PAPKE, E.: Synthese einiger Ketosäure-4-(*p*-azophenylphenyl)-semicarbazone und Ketosäure-4-(*p*-azophenylphenyl)-thiosemicarbazone und ihr papierchromatographisches Verhalten. *Pharmazie*, 18 (1963) 467–471.

RADECKE, H. R.: Empirische Darstellung der Beziehungen zwischen papierchromatographischem Verhalten und Struktur einiger physiologisch wichtiger, nichtflüchtiger aliphatischer Carbonsäuren. *Pharmazie*, 18 (1963) 402–405.

SCHMIDT, G. C., FISCHER, C. AND MCOWEN, J. M.: New method for location of organic acids on paper chromatograms. *J. Pharm. Sci.*, 52 (1963) 468–472 — α-naphthylamine with β-naphthol and α-naphthylamine with sulphanilamide and β-naphthol.

ZELENETSKAYA, A. A. AND NIKITINA, N. N.: (Paper chromatographic separation of ω-chlorocarboxylic acids and their determination). *Zh. Analit. Khim.*, 17 (1962) 1009–1014; *Anal. Abstr.*, 10 (1963) 4232.

13. STEROIDS

DEYL, Z., ROSMUS, J. AND PAVLÍČEK, M.: Some remarks on the paper "Centrifugally accelerated chromatography of steroids". *J. Chromatog.*, 11 (1963) 556–558.

KAUFMANN, H. P. AND VISWANATHAN, C. V.: Die Chromatographie des Cholesterins und seiner Ester. *Fette, Seifen, Anstrichmittel*, 65 (1963) 839–845.

PASQUALINI, J. R.: Formes d'élimination urinaire des hormones du groupe du cortisol et de la corticostérone après administration d'ACTH. *Bull. Soc. Chim. Biol.*, 45 (1963) 277–300.

ŠKÁRKA, B. AND PECIAR, J.: (Paper chromatography of keto steroids). *Chem. Zvesti*, 17 (1963) 449–450.

TAMM, CH., GUBLER, A., JUHASZ, G., WEISS-BERG, E. AND ZÜRCHER, W.: Über die Substratspezifität der Hydroxylierung von Androstan- und Pregnanderivaten durch *Fusarium lini*. *Helv. Chim. Acta*, 46 (1963) 889–906.

14. STEROID GLYCOSIDES

HOUK, A. E. H.: Gravimetric evaluation of paper chromatograms. II. Studies on ouabain, other glycosides, and prednisone. *J. Pharm. Sci.*, 52 (1963) 743–747.

LEWBART, M. L., WEHRLI, W., KAUFMANN, H. AND REICHSTEIN, T.: Die Cardenolide von *Gongronema gazense* (S. Moore) Bullock. *Helv. Chim. Acta*, 46 (1963) 517–529.

LEWBART, M. L., WEHRLI, W. AND REICHSTEIN, T.: Die Cardenolide von *Gongronema gazense* (S. Moore) Bullock. *Helv. Chim. Acta*, 46 (1963) 505–517 — chromatography on paper and in thin layers.

MAKSYUTINA, H. P.: (Cardiac glycosides of plants of the Cruciferae family *Cheiranthus allionii* hort). *Dokl. Akad. Nauk SSSR*, 150 (1963) 180–183.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BENASSI, C. A., PERISSINOTTO, B. AND ALLEGRI, G.: The metabolism of tryptophan in patients with bladder cancer and other urological diseases. *Clin. Chim. Acta*, 8 (1963) 822–831.

PAUMGARTNER, G., KRAUPP, O. AND FISCHER, F. X.: Papierchromatographische Auftrennung und Bestimmung von Kreatin und Kreatinin im Plasma. *Clin. Chim. Acta*, 8 (1963) 960–965.

ROBERTS, D. J.: The influence of lipid on the paper chromatographic behaviour of sympathomimetic catecholamines in plasma extracts. *J. Pharm. Pharmacol.*, 15 (1963) 579–583.

WAKSMUNDSKI, A. AND KACZOR, M.: (Separation of (±)-adrenaline into optically active isomers by paper chromatography). *Acta Polon. Pharm.*, 19 (1962) 143–147; *Anal. Abstr.*, 10 (1963) 1161 — (+)-camphor-10-sulphonic acid was added to the solution of adrenaline before chromatography.

18. AMINO ACIDS

CONTRACTOR, S. F.: Simple chromatographic separation of 5-hydroxytryptophan from tryptophan. *J. Chromatog.*, 11 (1963) 568–570.

HORISAKA, K.: Über das aus Hühnerharn isolierte Methylhistidin. *J. Biochem. (Tokyo)*, 54 (1963) 253–258.

ORME-JOHNSON, W. H. AND SKINNER, C. G.: Paper chromatography using liquid ion exchangers. *J. Chromatog.*, 11 (1963) 549–551 — good separation of aminodicarboxylic acids from their amides.

SCHRÖDER, I.: Untersuchungen zur Tryptophanbestimmung. *Pharmazie*, 18 (1963) 411–413 — quantitative analysis of ninhydrin–Cu complex after elution from paper.

SEN, N. P.: Synthesis of homotaurine. *Can. J. Chem.*, 40 (1962) 2189–2191; *C.A.*, 58 (1963) 6684e.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

SATAKE, M., OMORI, T., IWANAGA, S. AND SUZUKI, T.: Studies on snake venoms. XIV. Hydrolyses of insulin B chain and glucagon by proteinase C from *Agkistrodon halys blomhoffii* venom. *J. Biochem. (Tokyo)*, 54 (1963) 8–16.

SUZUKI, T., INOUYE, H., FUJIKAWA, K. AND NAGASAWA, S.: Studies on the chemical structure of colistin. II. Amino acid sequence of colistin A. *J. Biochem. (Tokyo)*, 54 (1963) 173–180.

TIUNOVA, N. A.: (R_F values of some dinitrophenylamino acids). *Tr. Gl. Botan. Sada, Akad. Nauk SSSR*, 8 (1961) 113; from *J. Chromatog., Chromatog. Data*, 11 (1963) D10.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

KLÖCKING, H. P. AND JOSCHKO, E.: (Paper-chromatographic detection of barbiturates by addition of bromine to the amide groups). *Z. Chem.*, 2 (1962) 373; *Anal. Abstr.*, 10 (1963) 4885.

SAITO, M., ISHIMOTO, N. AND ITO, E.: Uridine diphosphate N-acetylamino sugar derivatives in penicillin-treated *Staphylococcus aureus*. *J. Biochem. (Tokyo)*, 54 (1963) 273–278 — R_F values of 28 nucleotides.

22. ALKALOIDS

CLARKE, E. G. C. AND HAWKINS, A. E.: A note on the factors affecting R_F values on citrate buffered paper chromatograms. *J. Pharm. Pharmacol.*, 15 (1963) 390–393.

SINGH, C.: (The use of anionic dyes for the detection of polymeric antibiotics, alkaloids and other cationic compounds in paper chromatography). *Cesk. Farm.*, 12 (1963) 294–296 — 124 dyes were tested.

TOMITA, M. AND SUGAMOTO, M.: (Optical resolution of some alkaloids by paper chromatography and cellulose column chromatography). *Yakugaku Zasshi*, 82 (1962) 1141–1144; *C.A.*, 58 (1963) 6873d — development with buffer solutions.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

MELLMAN, W. J., BARNESS, L. A., REDESCO, T. A. AND BESSELMAN, D.: Indolylacroyl-glycine excretion in a family with mental retardation. *Clin. Chim. Acta*, 8 (1963) 843–847.

VIETTI-MICHELINA, M.: (Analytical separation of the 2- and 3-isomers of pyridinealdoxime, picolyl chloride and pyridylcarbinol N-oxide). *Rass. Chim.*, 14, No. 4 (1962) 144; *C.A.*, 58 (1963) 6195h.

24. ORGANIC SULPHUR COMPOUNDS

BRIDGES, J. W. AND WILLIAMS, R. T.: The metabolism of 5-*p*-aminobenzenesulfonamido-3-methylisothiazole (sulphasomizole). *J. Pharm. Pharmacol.*, 15 (1963) 565–573 — R_F values of 10 substances in 6 solvent systems.

HENTRICH, K.: Zur papierchromatographischen Unterscheidung von Sulfonamid-diuretica und Sulfonamiden anderer Indikationsgebiete. *Pharmazie*, 18 (1963) 405–409 — R_F values of 21 sulfonamides in 10 solvent systems.

TZAGOLOFF, A.: Metabolism of sinapine in mustard plants. I. Degradation of sinapine into sinapic acid and choline. *Plant Physiol.*, 38 (1963) 202–206.

25. ORGANIC PHOSPHORUS COMPOUNDS

DEUTSCH, E. AND FISCHER, M.: Die Verwendung eines stabilen Phospholipidpräparates als Trombocytenersatz bei Gerinnungsanalysen. *Arzneimittel-Forsch.*, 13 (1963) 439–445.

MURAKAMI, M., OZAWA, Y. AND FUNAHASHI, S.: Proteolipid from beef heart muscle. II. Chemical composition and subcellular distribution. *J. Biochem. (Tokyo)*, 54 (1963) 166–172.

26. METALLO-ORGANIC COMPOUNDS

PEDINELLI, M.: (Paper chromatography of alkyl-lead mixtures and of petrol containing these mixtures). *Chim. Ind. (Milan)*, 44 (1962) 651–652; *Anal. Abstr.*, 10 (1963) 680.

27. VITAMINS

CARDINI, C., CAVINA, G., CINGOLANI, E., MARIANI, A. AND VICARI, C.: Spectrophotometric and chromatographic examination of hydroxocobalamin. *Farmaco (Pavia), Ed. Prat.*, 17 (1962) 583–592; *Anal. Abstr.*, 10 (1963) 4423.

KNIEWALD, J. AND MILDNER, P.: (Determination of the vitamin-B complex in baker's yeast (*Saccharomyces cerevisiae*)). *Kem. Ind. (Zagreb)*, 11 (1962) 639–642; *Anal. Abstr.*, 10 (1963) 4421.

UEHARA, K., SUGENO, K. AND MIZOGUCHI, T.: Erythroflavin, threoflavin and their phosphoric esters. *J. Biochem. (Tokyo)*, 54 (1963) 267–272.

28. ANTIBIOTICS

ADDISON, E. AND CLARK, R. G.: The determination of epitetracycline and tetracycline by ion-exchange paper chromatography and its application to human urine and serum. *J. Pharm. Pharmacol.*, 15 (1963) 268–272.

BLINOV, N. O., YAKUBOV, G. Z., ARTAMONOVA, O. I. AND KHOKHLOVA, YU. M.: Paper-chromatographic isolation of antibiotics of the mycetin-violarin group). *Antibiotiki*, 7 (1962) 1063–1067; *Anal. Abstr.*, 10 (1963) 4360.

CANTERELLI, G.: (Separation of tetracycline antibiotics by paper chromatography). *Farmaco (Pavia), Ed. Prat.*, 17 (1962) 728–731; *Anal. Abstr.*, 10 (1963) 4358.

29. INSECTICIDES AND OTHER PESTICIDES

GRAHAM, S. O.: Indophenol blue as a chromogenic agent for identification of halogenated aromatic hydrocarbons. *Science*, 139 (1963) 835–836; *Anal. Abstr.*, 10 (1963) 4945.

30. SYNTHETIC AND NATURAL DYES

GASPARIČ, J. AND MATRKA, M.: (Paper chromatography of nigrosines). *Chem. Prumysl*, 13 (1963) 22–23; *Anal. Abstr.*, 10 (1963) 4770.

KIEL, E. G. AND KUYPERS, G. H. A.: (Paper chromatography of dyestuffs. IV. Vat dyestuffs). *Tex*, 22 (1963) 162–165; *Anal. Abstr.*, 10 (1963) 4771.

ŠRÁMEK, J.: Paper chromatography of dyes. II. Paper chromatography of vat and sulphur dyes. *J. Chromatog.*, 11 (1963) 524–533.

ŠRÁMEK, J.: (Chromatography of synthetic dyestuffs. II. Paper chromatography of water-soluble dyestuffs). *Sb. Veda Vyzkum Prumyslu Textilnim*, 6 (1963) 99–129.

31. PLASTICS AND THEIR INTERMEDIATES

ŠRÁMEK, J. AND LAŠ, L.: (Paper chromatography of synthetic resins. I. Qualitative determination of thermosetting resins by means of paper chromatography). *Sb. Veda Vyzkum Prumyslu Textilnim*, 6 (1963) 87–97.

WEIGEL, K.: (Paper-chromatographic analysis of synthetic resins). *Plaste Kautschuk*, 9 (1962) 123–126; *Anal. Abstr.*, 10 (1963) 1089.
WEIGEL, K.: (Examination of developing liquids for the paper-chromatographic analysis of paint resins). *Deut. Farben-Z.*, 16 (1962) 297–303, 339–347; *Anal. Abstr.*, 10 (1963) 4278.

32. PHARMACEUTICAL APPLICATIONS

BOBRANSKI, B. AND SYPER, L.: Identification of drugs containing unsaturated groups by paper chromatography. *Microchem. J., Symp. Ser.*, 2 (1962) 749–756; *C.A.*, 58 (1963) 6647a — Hg adducts.
NEDERGAARD, M.: (Simple paper chromatographic routine analysis for some galenicals, adapted for use in pharmacies). *Arch. Pharm. Chemi*, 69 (1962) 745–750; *C.A.*, 58 (1963) 6646c.
PUSZ, A. AND BÜCHNER, S.: The chromatographic examination of *Adonis vernalis* L. *Arzneimittel-Forsch.*, 13 (1963) 409–412.
WOLLMANN, CHR. AND SCHULZ, D.: Papierchromatographischer Nachweis von Pentinol® und Dormutil®. *Pharmazie*, Beilage No. 7, *Pharm. Praxis*, 7 (1963) 128–130.

33. INORGANIC SUBSTANCES

BARCZA, L. AND MESTER, O.: (Sensitive and selective detection of alkaline earth metals with phthalein complexon). *Ann. Univ. Sci. Budapest. Rolando Eotvos Nominatae, Sect. Chim.*, 4 (1962) 33–35; *C.A.*, 58 (1963) 6172b.
DEY, A. K. AND SINGH, E. J.: Circular paper-chromatographic separation of microamounts of metal ions using Weisz ring oven technique. *Microchem. J., Symp. Ser.*, 2 (1962) 771–779; *C.A.*, 58 (1963) 6165d.
ELBEIH, I. I. AND GARBA, G. G.: Determination of zirconium with hafnium in zircon via paper-chromatographic separation. *Chemist-Analyst*, 52 (1963) 13–14; *Anal. Abstr.*, 10 (1963) 4611.
GÁNTI, T. AND NOVÁK, E.: (General method for the development of paper chromatograms. I. Detection of inorganic ions). *Magy. Kem. Folyoirat*, 68 (1962) 293–296; *Anal. Abstr.*, 10 (1963) 928 — benzidine + furfuraldehyde.
GRASSINI, G. AND ALBERTI, G.: A very sensitive fluorescence test for uranium and its use in the paper-chromatographic estimation of it in natural waters. *Microchem. J., Symp. Ser.* 2 (1962) 285–291; *C.A.*, 58 (1963) 6573e.
KRZECZKOWSKA, I. AND WIDOMSKA-CZEKAJSKA, T.: (Paper chromatographic detection of cobalt in dilute solutions). *Ann. Univ. Mariae Curie-Sklodowska, Lublin-Polonia, Sect. D*, 16 (1961) (publ. 1962) 307–321; *C.A.*, 58 (1963) 6172d.
LABIĬ, YU. M.: (Detection of arsenic, antimony and tin when present together and with other cations by precipitation chromatography on paper). *Ukr. Khim. Zh.*, 28 (1962) 641–643; *Ref. Zh., Khim.*, 19GDE (1963) Abstr. No. 8G49; *Anal. Abstr.*, 10 (1963) 4549.
LIIMATTA, A. M. AND SPAIN, J. D.: Precipitation chromatography. Separations of metals as ferrocyanides on filter paper. *Anal. Chem.*, 35 (1963) 1898–1900.
OHASHI, S. AND VAN WAZER, J.: Paper chromatography of very long chain phosphates. *Anal. Chem.*, 35 (1963) 1984–1985.
POLUEKTOV, N. S. AND NIKONOVA, M. P., in D. I. RYABCHIKOV (Editor): *Rare-Earth Elements (Extraction, Analysis, Applications)*, Academy of Science, USSR, Moscow, 1959; translated from Russian, published for the National Science Foundation, Washington D.C. and the Department of Commerce by the Israel Program for Scientific Translation, 1960, p. 227; from *J. Chromatog., Chromatog. Data*, 11 (1963) D17 — R_F values of rare earth metal ions.
TZOU, S. F. AND TAO, P. L.: Paper-chromatographic separation of zirconium and hafnium. *Acta Chim. Sinica*, 28 (1962) 344–346; *Anal. Abstr.*, 10 (1963) 4116.
ZAV'YALOV, V. A. AND MAL'TSEVA, O. S.: (Application of paper chromatography to the study of sedimentary rocks). *Novosti Neft. i Gaz. Tekhn. Geol.*, (1962) 32–35; *Ref. Zh. Khim.*, 19GDE (1963) Abstr. No. 6G124; *Anal. Abstr.*, 10 (1963) 4706.

34. RADIOACTIVE COMPOUNDS

MCWEENY, D. J. AND BURTON, H. S.: Simple radio-chromatogram scanner. *J. Sci. Instr.*, 39 (1962) 398; *Anal. Abstr.*, 10 (1963) 858.
OSINSKI, P.: Synthesis of T-labelled adrenal and gonadal hormones. *Tritium Phys. Biol. Sci., Proc. Symp. Detection Use, Vienna, Austria, 1961*, Vol. 2 (publ. 1962) 113–119; *C.A.*, 58 (1963) 6884a.

Thin-layer Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

HONEGGER, C. G.: Einfluss der Kammersättigung in der Dünnschichtchromatographie. *Helv. Chim. Acta*, 46 (1963) 1730–1734.

PATAKI, G. AND KELLER, M.: Einfluss der Schichtdicke auf die R_F-Werte in der Dünnschichtchromatographie. *Helv. Chim. Acta*, 46 (1963) 1054–1056.

3. TECHNIQUES I

KNAPPE, E., PETERI, D. AND ROHDEWALD, I.: Imprägnierung chromatographischer Dünnschichten mit Polystyren. Trennung und Identifizierung substituierter 2-Hydroxybenzophenone und anderer UV-Absorber. *Z. Anal. Chem.*, 197 (1963) 364–369.

4. TECHNIQUES II

HONEGGER, C. G.: Neuere Untersuchungen zur präparativen Dünnschichtchromatographie. *Helv. Chim. Acta*, 46 (1963) 1772–1776.

6. ALCOHOLS

NAFF, M. B., NAFF, A. S. AND STRITE, J. A.: Separation of 2-butyne-1,4-diol, its monoesters and diesters. A comparison of glass-paper chromatography and thin-layer chromatography. *J. Chromatog.*, 11 (1963) 496–503 — 7 advantages of thin layers.

10. CARBOHYDRATES

HAY, G. W., LEWIS, B. A. AND SMITH, F.: Thin-film chromatography in the study of carbohydrates. *J. Chromatog.*, 11 (1963) 479–486.

13. STEROIDS

HARA, S. AND TAKEUCHI, M.: A systematic analysis of bile acids and their derivatives by thin-layer chromatography. *J. Chromatog.*, 11 (1963) 565–567 — R_F values of 57 substances.

HARKE, H. P. AND VOGEL, P.: Beitrag zum Nachweis von tierischen Fetten in Pflanzenfetten. *Fette, Seifen, Anstrichmittel*, 65 (1963) 806–809 — thin-layer and paper chromatography of sterols.

USUI, T.: Thin-layer chromatography of bile acids with special reference to separation of keto bile acids. *J. Biochem. (Tokyo)*, 54 (1963) 283–286 — R_F values of 14 bile acids.

14. STEROID GLYCOSIDES

TAKEDA, K., HARA, S., WADA, A. AND MATSUMOTO, N.: A systematic, simultaneous analysis of steroid sapogenins by thin-layer chromatography. *J. Chromatog.*, 11 (1963) 562–564 — 20 genins.

18. AMINO ACIDS

KELLER, M. AND PATAKI, G.: Freie Aminosäuren im menschlichen Ejakulat. 1. Mitt. Dünnschichtchromatographischer Nachweis. *Helv. Chim. Acta*, 46 (1963) 1687–1691.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

SCHWYZER, R., COSTOPANAGIOTIS, A. AND SIEBER, P.: Zwei Synthesen des α-Melanotropins (α-MSH) mit Hilfe leicht entfernbarer Schutzgruppen. *Helv. Chim. Acta*, 46 (1963) 870–889 — thin-layer and paper chromatography.

22. ALKALOIDS

GOVINDACHARI, T. R., NAGARAJAN, K. AND SCHMID, H.: Umlagerungsreaktionen des Kopsins. *Helv. Chim. Acta*, 46 (1963) 433–444 — R_F values of 13 kopsin derivatives.

24. ORGANIC SULPHUR COMPOUNDS

BIĆAN, T. F. AND KAJGANOVIĆ, V.: Separation and identification of sulphonamides by thin-layer chromatography. *J. Chromatog.*, 11 (1963) 492–495.

25. ORGANIC PHOSPHORUS COMPOUNDS

HANAHAN, D. H., EKHOLM, J. AND JACKSON, C. M.: Studies on the structure of glyceryl ethers and the glyceryl ether phospholipids of bovine erythrocytes. *Biochemistry*, 2 (1963) 630–641.

HAUSHEER, L., PEDERSEN, W. AND BERNHARD, K.: Ein Verfahren zur quantitativen Isolierung reiner Sphingomyeline aus Menschen- und Rattenhirn. *Helv. Chim. Acta*, 46 (1963) 601–604.

30. SYNTHETIC AND NATURAL DYES

STIER, A. AND SPECHT, W.: Chromatographische Prüfung von Xanthenfarbstoffe für die histologische Färbung nach Unna-Pappenheim. *Naturwiss.*, 50 (1963) 549.

31. PLASTICS AND THEIR INTERMEDIATES

HAUB, H. G. UND KÄMMERER, H.: Dünnschichtchromatographie von struktur- und molekulareinheitlichen *p*-Kresol–Formaldehyd-Kondensaten. *J. Chromatog.*, 11 (1963) 487–491.

33. INORGANIC SUBSTANCES

RÖSSEL, T.: Die chromatographische Analyse von Phosphaten. II. Die Dünnschichtchromatographie der kondensierten Phosphate. *Z. Anal. Chem.*, 197 (1963) 333–347.

34. RADIOACTIVE COMPOUNDS

SHEPPARD, H. AND WEN HUI TSIEN: Autoradiography of tritium-containing thin layer chromatograms. *Anal. Chem.*, 35 (1963) 1992.

Gas Chromatography

1. REVIEWS AND BOOKS

ETTRE, L. S.: Fourth Symposium on Gas Chromatography, Eastern Germany, May, 1963. *J. Gas Chromatog.*, 1, No. 10 (1963) 15–19 — a review of 32 of the 46 papers read at the Symposium.

ETTRE, L. S.: Sixth World Petroleum Congress, Frankfurt am Main, June 19–26, 1963. *J. Gas Chromatog.*, 1, No. 9 (1963) 23–24 — a review of 7 GC papers.

ETTRE, L. S.: Italian meeting on "Immediate Separation and Chromatography", Milan, June 14–16, 1963. *J. Gas Chromatog.*, 1, No. 9 (1963) 25 — a review of 11 GC papers.

MILLER, J. T. AND INST, F. P. Quality control instrumentation. Part. I. Gas–liquid chromatography. *Instr. Pract.*, 17 (1963) 627–637 — 12 references.

MIWA, T. K.: Identification of peaks in gas–liquid chromatography. *J. Am. Oil Chemists' Soc.*, 40 (1963) 309–313 — a review of fatty acid identification methods based on retention data.

SCHOEDLER, C.: Les applications industrielles de la chromatographie en phase gazeuse. *Genie Chim.*, 89, No. 5 (1963) 146–161 — a general review.

TADMOR, J.: Gas chromatography in inorganic chemistry — in M. LEDERER (Editor): *Chromatographic Reviews*, Vol. 5, Elsevier, Amsterdam, 1963, pp. 223–235 — a review with 109 references.

WILLIAMS, M. C. AND REISER, R.: The chemical and biological assay of essential fatty acids. *J. Am. Oil Chemists' Soc.*, 40 (1963) 237–241 — a review with 69 references on chromatographic methods including GC.

2. FUNDAMENTALS, THEORY AND GENERAL

2b. Gas–solid systems

ZHUKHOVITSKIĬ, A. A., TURKEL'TAUB, N. M., MALYASOVA, L. A., SHLYAKHOV, A. F., NAUMOVA, V. V. AND POGREBNAYA, T. I.: (Chromatography without carrier gas). *Zavodsk. Lab.*, 29 (1963) 1162–1166 — GSC of frontal type.

2c. Thermodynamics and theoretical relationships

BLAUSTEIN, B. D., ZAHN, C. AND PANTAGES, G.: Temperature as a factor in determining order of elution in gas–liquid chromatography of some C_5–C_8 hydrocarbons. *J. Chromatog.*, 12 (1963) 104–106 — log of relative retention *versus* $1/T$ plots for alkanes and cycloalkanes are discussed.

BURNETT, M. G.: Determination of partition coefficients at infinite dilution by the gas chromatographic analysis of the vapor above dilute solutions. *Anal. Chem.*, 35 (1963) 1567–1570 — partition coefficients for acetone and C_1–C_4 alcohols from water at 25° and for C_5–C_8 hydrocarbons from squalane at 80°.

CATES, V. E. AND MELOAN, C. E.: Compound identification by gas chromatography. A comparison to the paper chromatography method of Schauer and Bulirsch. *J. Chromatog.*, 12 (1963) 15–21 — analogic use of R_M values in GC-identification of aliphatic amines is shown.

EVANS, M. B.: Gas–liquid chromatography in qualitative analysis. Part V. The determination of relative retentions in R_{x_9} and retention index units by means of secondary standards. *J. Chromatog.*, 12 (1963) 2–7 — indexing by means of internal standards other than *n*-alkanes.

LIBERTI, A., CARTONI, G. P. AND BRUNER, F.: Isotopic effects in gas–liquid chromatography. I. Gas chromatographic behaviour of benzene and perdeuterobenzene. *J. Chromatog.*, 12 (1963) 8–14 — a study of thermodynamics of the chromatographic process; separation is achieved on squalane-coated glass capillary ($r_{1,2} = 1.0357$).

2d. General

DUŠKOVÁ, L., GRUBNER, O., HANUŠ, V., KÖSSLER, I. AND MATYSKA, B.: (Selection of extraction agents for isoprene rectification). *Chem. Prumysl*, 13 (1963) 513–516 — relative volatilities of isoprene, trimethylethylene and 2-methylbutene-1 in 34 liquids.

NELSON, D. C. AND PAULL, D. L.: Absolute calibration of gas chromatographic apparatus from ion-chamber measurements. *Anal. Chem.*, 35 (1963) 1571–1574 — an ion chamber is connected in series with a gas chromatograph; the effect of various peak shapes on the signal of an equivalent fraction of radioactivity is measured within 1% of accuracy.

SAID, A. S. AND ROBINSON, M. A.: Optimum points for the measurement of the number of theoretical plates and peak areas in G.C. *J. Gas Chromatog.*, 1, No. 9 (1963) 7–11 — optimum is equal to e^{-1} height.

SHANIN, M. M. AND LIPSKY, S. R.: The role of argon metastable atoms in the ionization of organic molecules. *Anal. Chem.*, 15 (1963) 1562–1566 — vapour concentration 5–10 vol. % in carrier gas may exist without any overloading phenomena.

3. TECHNIQUES I

3a. Detectors

GUILLEMIN, C. L. AND AURICOURT, F.: Gas phase chromatography. Study of the Gow-Mac gas density balance. Application to quantitative analysis. *J. Gas Chromatog.*, 1, No. 10 (1963) 24–29 — quantitative calibrations were found to be necessary in some cases.

MCFADDEN, W. H. AND TERANISHI, R.: Fast-scan mass spectrometry with capillary gas–liquid chromatography in investigation of fruit volatiles. *Nature*, 200 (1963) 329–330 — high-speed oscillograph with a scan rate of mass 24–200 in 6 sec; recorder with time constant 10^{-3} sec; temperature of the line entering the mass spectrometer is maintained at that of the GC column.

NERHEIM, A. G.: A gas-density detector for gas chromatography. *Anal. Chem.*, 35 (1963) 1640–1644 — equations were developed for the interrelationship of change in density, flow and electrical response; correction of 5% is needed for hydrogen only.

3b. Column performance and filling studies

AL'TSHULER, O. V., VINOGRADOVA, O. M., ROGINSKIĬ, S. Z. AND CHIRKOV, YU. N.: (Possibility of chromatographic separation on gas–liquid columns without inert carrier gas). *Dokl. Akad. Nauk SSSR*, 152 (1963) 893–896 — a compound that is well soluble acts as carrier gas (pseudodisplacement).

HUNTER, I. R. AND WALDEN, M. K.: Column rejuvenation in gas chromatography by means of reservoir sections. *Anal. Chem.*, 35 (1963) 1765 — a short precolumn is mounted between the carrier gas inlet and the main column.

KUBRYAVTSEVA, N. A., TARASOV, A. I. AND SHIPANOVA, A. I.: (About the quality of the vaseline grease during the modification of TZK for the chromatographic separation of hydrocarbons). *Khim. i Tekhnol. Topliv i Masel*, No. 10 (1963) 59–62 — TZK is a diatomaceous earth (T) of Zikeevsk's (Z) quarry (K).

MACDONALD, R. AND BRUNET, P. E.: Modification of gas chromatographic columns by addition of glycerol. *J. Chromatog.*, 12 (1963) 266–267 — 1% glycerol acts as tailing reducer.

MATSEN, J. M. AND HARDING, J. W.: Theoretical plate height in helical chromatographic columns. *J. Chromatog.*, 12 (1963) 145–149 — the theoretical plate number in coiled columns is given by the ratio of the second power of the radius of the coil and the radius of the column.

PAYN, D. S., REARDON, W. D. AND HARVEY, L. J.: Rejuvenation of gas–liquid chromatography columns *in situ*. *Nature*, 200 (1963) 467 — injection of stationary phase solution into the column.

STEWART, G. H. AND KELLER, R. A.: Changes occurring with the immobile liquid phase in gas–liquid chromatography. III. The effect on observed plate height. *J. Chromatog.*, 12 (1963) 150–162 — HETP is influenced by non-uniform distribution of the stationary liquid in different column sections.

VANDENHEUVEL, W. J. A., GARDINER, W. L. AND HORNING, E. C.: Preparation of thin-film gas chromatographic columns with polyvinylpyrrolidone-inactivated supports. *Anal. Chem.*, 35 (1963) 1745–1746 — 1–2% coating gives a good support for steroid separations.

3c. Apparatus, accessories and materials for GC

AST, H. J.: Needle cup sampling device for gas chromatography. *Anal. Chem.*, 35 (1963) 1764–1765.

BECKMAN, H. AND BEVENUE, A.: A one-piece all-glass injection block and gas chromatographic column for the microanalysis of halogenated hydrocarbons. *J. Chromatog.*, 12 (1963) 109–111 — a photo of the apparatus is given; recoveries of 74–100% for 9 pesticides are obtained.

BROWN, P. M. AND O'MALLEY, D. R.: Method and apparatus for analyzing fuel–air mixtures. *U.S. Pat.*, 3,096,157, June 2, 1963 — CO_2–H_2O–N_2 mixtures are analysed on a dioctyl phthalate column.

DUTTON, H. J.: Monitoring gas chromatography for H^3 and C^{14}-labeled compounds by liquid scintillation counting. *Advances in Tracer Methodology*, Vol. 1, Plenum Press, New York, 1963, pp. 147–152 — accessories for condensation from GC streams for scintillator solvent.

FRAADE, D. J.: A successful sample system for sulphur plant chromatographic analysis. *Process Control Automation*, 10 (1963) 242–244.

GREEN, Jr., L. E.: Gas analysis device. *U.S. Pat.*, 3,095,278, June 25, 1963 — a portable gas chromatographic apparatus with a flame ionization detector.

HOLM-JENSEN, I.: An improved gas absorption device for conductometric micro-determination of carbon dioxide. *Anal. Chim. Acta*, 29 (1963) 356–368 — also applicable for GC.

HORTON, A. D. AND MEYER, A. S.: Gas chromatographic analysis of radioactive samples. *U.S. Gov. Res. Rep.*, ORNL-3397 UC-4 (1963) 48 — apparatus for off-gases from the dissolution of uranium carbides in water.

MCEWEN, D. J.: Temperature programmed capillary columns in gas chromatography. *Anal. Chem.*, 35 (1963) 1636–1640 — applied to dilute hydrocarbon gas mixtures (99.8% N_2); retention data for 67 C_5–C_8 hydrocarbons are given within ± 1.8% accuracy.

NELSON, D. C., RESSLER, Jr., P. C. AND HAWES, R. C.: Performance of an instrument for simultaneous gas chromatographic and radioactivity analysis. *Anal. Chem.*, 35 (1963) 1575–1579 — construction details and examples of separation of labelled amino acids, fatty acids and steroids are given.

TAYLOR, B. W. AND POLI, Jr., A. A.: Apparatus for gas analysis. *U.S. Pat.*, 3,097,518, July 16, 1963 — thermal conductivity detector with 4 thermistors.

VAN SWAAY, M.: The design of an automatic absolute flow meter for gas chromatography. *J. Chromatog.*, 12 (1963) 99–103 — constructive schemes are given; the use of the flow meter as a detector is discussed.

WOLFGANG, R. L.: Vapor phase proportional counting. *Advances in Tracer Methodology*, Vol. 1, Plenum Press, New York, 1963, pp. 183–184 — high-boiling compounds (265°) were analysed by combination of PTGC and heated counters.

4. TECHNIQUES II

4a. Preparative-scale GC

GUILLEMIN, C. L.: A novel technique for filling wide diameter gas chromatographic columns. *J. Chromatog.*, 12 (1963) 163–166 — a fluidization technique gives uniform density of filling along and across the column diameter; 315 TP/m for 6 cm I.D. column and 1,2 ml sample of C_5H_{12} and C_6H_{14} (1:1) were obtained.

4d. Special mictotechniques

FRANC, J. AND MICHAJLOVA, S.: Identifizierung organischer Substanzen mit Hilfe der Gaschromatographie (chromatographische Spektren). *J. Chromatog.*, 12 (1963) 22–31 — the method is based on eluting one compound simultaneously from four columns with different stationary phases through one detector.

GAZIEV, G. A., FILIPOVSKIĬ, V. YU. AND YANOVSKIĬ, M. I.: (Kinetics of heterogenous catalytic reactions with impulsing chromatographic regime in the conditions of ideal linear chromatography). *Kinetika i Kataliz*, 4 (1963) 688–697.

JANÁK, J.: Gas chromatography as a sampling procedure for thin-layer or paper chromatography. *J. Gas Chromatog.*, 1, No. 10 (1963) 20–23 — new multi-dimensional chromatography using different chromatographic methods; the gas chromatogram is exposed on the starting line of a thin layer or a sheet of paper.

LYSYJ, I. AND GREENOUGH, R. C.: Gas chromatographic technique for determination of hydridic and active hydrogen in organic materials. Microanalysis of borane compounds and alcohols. *Anal. Chem.*, 35 (1963) 1657–1659 — determination of H_2 liberated during chemical reactions.

NAKANISHI, J. AND TAMARU, K.: Study of the mechanism of the water-gas-shift reaction on an iron oxide catalyst using a gas chromatographic technique. *Trans. Faraday Soc.*, 59 (1963) 1470–1471 — H_2 + CO_2 were introduced into the column and their adsorption on the catalyst was measured from retention times.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

MIYAKE, H. AND MITOOKA, M.: Gas-chromatographic analysis of C_1–C_4 hydrocarbons with mixed stationary liquid of Apiezon–dibutyl maleate. *J. Chem. Soc. Japan*, 84 (1963) 593–595 (Engl. summary, A39).

5b. Other hydrocarbons

ACKMAN, R. G.: 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. *J. Chromatog.*, 12 (1963) 271–276 — retention data of 16 *cis*- and *trans*-isomers of C_5–C_7 monoalkenes on 5 stationary phases at 50° and 100°.

BLAKE, Jr., C. A.: Separation of diethylbenzenes by gas chromatography using packed columns. *Anal. Chem.*, 35 (1963) 1759–1760 — separation of *o*-, *m*- and *p*-isomers on Bentone 34 modified with silicone oil by PTGC 70/150°.

FORD, D. C.: Analysis of light hydrocarbons, C_2 to C_5. *J. Gas Chromatog.*, 1, No. 8 (1963) 36 — retention data of 21 C_2–C_5 hydrocarbons on DOW P-200 and dimethylsulpholane at 30°.

HORTON, A. D.: Gas chromatographic determination of products of dissolution of uranium carbides in water. *U.S. Gov. Res. Rep.*, ORNL-3397 UC-4 (1963) 42–47 — 26 hydrocarbons on di-2-ethylhexyl sebacate and squalane by PTGC.

LIJINSKY, W. AND MASON, G.: Programmed temperature gas chromatography of tars and pyrolysates of naphthalenes at moderate temperatures. *J. Gas Chromatog.*, 1, No. 9 (1963) 12–16 — some polynuclear hydrocarbons were identified on SE-30 at programmed temperature 100–220°.

MOSELY, R. B. AND ARCHIBALD, R. C.: Adsorption of hydrocarbons on acidic oxides as measured chromatographically. *J. Catalysis*, 2 (1963) 131–137 — GSC has been used to measure the relative adsorption ability of hydrocarbons on silica–alumina and silica–magnesia.

SOJÁK, L., GREGORÍK, M. AND KURCOVÁ, J.: (Separation of low-boiling gasolines by means of gas chromatography). *Ropa Uhlie*, 5 (13) (1963) 289–293 — C_1–C_6 hydrocarbons on squalane at 29° and 50°.

SOLO, A. J. AND PELLETIER, S. W.: Gas liquid chromatography of phenanthrenes. Use in identification of the components of mixtures of alkyl phenanthrenes. *Anal. Chem.*, 35 (1963) 1584–1587 — retention data of 60 alkyl derivatives on SE-30, polar QF-1-0065 and 5 other stationary phases at 215–295°.

WELLS, A. W., NORMAN, S. M. AND ATROPS, E. P.: Measurement of biphenyl vapor. *J. Gas Chromatog.*, 1, No. 9 (1963) 19–20 — biphenyl from the atmosphere of fruit storehouses in amounts of μg/l of air.

6. ALCOHOLS

HAKEN, J. K.: The gas chromatographic analysis of alcohols using a peak shifting technique. *J. Gas Chromatog.*, 1, No. 10 (1963) 30 — GC separation on SE-30 after conversion to acetates.

7. PHENOLS

KOLLOF, R. H., BREUKLANDER, L. J. AND BARKLEY, L. B.: Gas chromatographic analysis of chlorophenol mixtures. *Anal. Chem.*, 35 (1963) 1651–1654 — retention data of 7 derivatives on Carbowax 20M at 200° and SE-30 at 172°.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BLACK, L. T. AND EISENHAUER, R. A.: Determination of cyclic fatty acids by gas–liquid chromatography. *J. Am. Oil Chemists' Soc.*, 40 (1963) 272–276 — on DEGS at 220°.

CALZOLARI, C. AND CERMA, E.: (Fatty substances of coffee). *Riv. Ital. Sostanze Grasse*, 40 (1963) 176–180 — on PEGS at 205°.

EDWARDS, H. M. AND MARION, J. E.: A simple method of calibrating a gas liquid chromatography column for quantitative fatty acid analysis. *J. Am. Oil Chemists' Soc.*, 40 (1963) 299–300 — an equation for calculating corrections to molar response is given.

FIRESTONE, D.: The determination of polymers in fats and oils. *J. Am. Oil Chemists' Soc.*, 40 (1963) 247–255 — methyl esters of polymerized fats on SE-52 at 296°.

GAST, L. E., SCHNEIDER, W. J., FOREST, C. A. AND COWAN, J. C.: Composition of methyl esters from heat-bodied linseed oils. *J. Am. Oil Chemists' Soc.*, 40 (1963) 287–289 — on PEGS at 170°.

HAWKE, I. C.: The distribution of fatty acids between the α- and β-positions of the glycerophospholipids of buttermilk. *J. Lipid Res.*, 4 (1963) 255–259 — methyl esters above C_{18} on Apiezon L at 225°.

HOCHAPFEL, A. AND PAQUOT, C.: Dérivés α-substitués des acides aliphatiques normaux à longue chaîne. VI. Étude des alkyl-2-tétrahydropyranones-3. *Oléagineaux*, 18 (1963) 457–470 — GC used for identification.

JAKUBOWSKI, A., SOBIERAJSKA, I. AND MODZELEWSKA, K.: (Comparison of fatty acid composition of polymerized rape-oils from the inland polymerization plants). *Prace Inst. Lab. Badawczych Przemyslu Spożywczego*, 13, No. 1 (1963) 41–57 — on DEGS at 208°.

KABOT, F. J. AND ETTRE, L. S.: Gas chromatography of free fatty acids. II. Packed columns with inert support material. *J. Gas Chromatog.*, 1, No. 9 (1963) 17–18 — porous Teflon 6 is used up to C_{14} acids.

KABOT, F. J. AND ETTRE, L. S.: Gas chromatography of free fatty acids. III. Quantitative aspects. *J. Gas Chromatog.*, 1, No. 10 (1963) 7–10 — relative molar and weight responses (flame ionization) for C_2–C_{18} fatty acids are given and discussed.

LITCHFIELD, C., REISER, R. AND ISBELL, A. F.: The analysis of *cis-trans* fatty acid isomers using gas liquid chromatography. *J. Am. Oil Chemists' Soc.*, 40 (1963) 302–309 — retention data from capillary columns with Apiezon L or DEGS at 168–200°.

MIWA, T. K., EARLE, F. R., MIWA, G. C. AND WOLF, I. A.: Fatty acid composition of maturing *Vernonia anthelmintica* (L.) willd. seeds. Dihydroxyoleic acid — a possible precursor of epoxyoleic acid. *J. Am. Oil Chemists' Soc.*, 40 (1963) 225–229.

NESTLER, F. H. M. AND ZINKEL, D. F.: Separation of the methyl esters of resin acids by gas liquid chromatography. *Anal. Chem.*, 35 (1963) 1747–1749 — retention data of 7 resin acid methyl esters (from pimarate to neoabietate) on DEGS at 200°.

PRIVETT, O. S., BLANK, M. L. AND ROMANUS, O.: Isolation analysis of tissue fatty acids by ultramicro-ozonolysis in conjunction with thin-layer chromatography and gas–liquid chromatography. *J. Lipid Res.*, 4 (1963) 260–265.

ROBB, E. W. AND WESTBROOK, III, J. J.: Preparation of methyl esters for gas liquid chromatography of acids by pyrolysis of tetramethylammonium salts. *Anal. Chem.*, 35 (1963) 1644–1647 — esters are formed by injection into a vaporizer heated to 330–365°; only oxalic, malonic, malic and citric acid do not react.

SALLEE, E. M.: Report of the Spectroscopy Committee A.O.C.S. 1961–1962, Gas Chromatography Subcommittee. *J. Am. Oil Chemists' Soc.*, 40, No. 3 (1963) XIV — statistical report on optimal methods for analytical esterification of fatty acids for GC.

SCHOLFIELD, C. R., JONES, E. P., BUTTERFIELD, R. O. AND DUTTON, H. J.: Argentation in countercurrent distribution to separate isologues and geometric isomers of fatty acid esters. *Anal. Chem.*, 35 (1963) 1588–1591 — *cis-*, *trans* (*trans*, *cis*), *cis,cis* and *trans,trans* isomers were separated on Apiezon L, 200 ft. capillary column at 200°.

SHELLEY, R. N., SALWIN, H. AND HORWITZ, W.: Quantitative determination of formic, acetic, propionic and butyric acids by gas chromatography. *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 486–493 — retention data on 4 stationary phases at 4 temperatures.

12. ORGANIC PEROXIDES

CULLIS, C. F. AND FERSHT, E.: The analysis of volatile organic peroxides by gas–liquid chromatography. *Combust. Flame*, 7 (1963) 185–193 — retention data of tertiary, secondary and primary alkyl monohydroperoxides from isopentane on silicone oil and dinonyl phthalate at 56°, 76° and 100°.

13. STEROIDS

COX, R. I.: Gas chromatography in the analysis of urinary pregnanediol. *J. Chromatog.*, 12 (1963) 242–245 — retention times of 10 steroids at 220 and 240°.

EISNER, J. AND FIRESTONE, D.: Gas chromatography of unsaponifiable matter. II. Identification of vegetable oils by their sterols. *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 542–550 — on SE-52 at 219°.

JELLIFFE, R. W. AND BLANKENHORN, D. H.: Gas chromatography of digitoxigenin. *J. Chromatog.*, 12 (1963) 268–270 — trimethylsiloxy derivatives are separated on SE-30 on siliconized support material at 228°.

15. TERPENE DERIVATIVES

BUTTERY, R. G., MCFADDEN, W. H., TERANISHI, R., KEALY, M. P. AND MON, T. R.: Constituents of hop oil. *Nature*, 200 (1963) 435–436 — 50 compounds identified by capillary PTGC and mass spectrometry.

CAPELLA, P., FEDELI, E., CIRIMELE, M. AND JACINI, G.: (The Fitelson's reaction: Identification of the structure of tea-oil). *Riv. Ital. Sostanze Grasse*, 40 (1963) 296–299 — reaction gives butyrospermol.

FERNANDEZ, B., FAGERSON, I. S. AND NAWARA, W. W.: Applicability of gas chromatography to detection of changes in orange oil. *J. Gas Chromatog.*, 1, No. 9 (1963) 21–22 — retention data for 23 terpene derivatives on 2 phases in capillary columns.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

NEUBAUER, N. R., SKRECKOSKI, G. R., WHITE, R. G. AND KANE, A. J.: Gas chromatographic determination of free toluene diisocyanate in adducts with trimethylolpropane. *Anal. Chem.*, 35 (1963) 1647–1649 — for range 0.5 to 10% GC analysis is better than chemical analysis.

18. AMINO ACIDS

MAKISUMI, S., NICHOLAS, C. H. AND SAROFF, H. A.: The influence of esterifying and acetylating groups on the retention times of amino acid derivatives in gas chromatography. *J. Chromatog.*, 12 (1963) 106–108 — retention data on neopentyl glycol succinate on Fluoropak 80 at 135–180°.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

VIETTI-MICHELINA, M. AND PILLERI, R.: Gaschromatographie der Isomeren Pyridin-2- und -4-carbonsäure-*n*-butylester. *Z. Anal. Chem.*, 196 (1963) 268–270.

26. METALLO-ORGANIC COMPOUNDS

FRITZ, G. AND KSINSIK, D.: Bildung siliciumorganischer Verbindungen. XXI. Die Bildung chlorierter Silicium-methylene bei der Pyrolyse der Methylchlorsilane. *Z. Anorg. Allgem. Chem.*, 322 (1963) 46–57.

FRITZ, G. AND SONNTAG, G.: Bildung siliciumorganischer Verbindungen. XX. Über eine Umlagerung im Si–C–Si-Gerüst bei der Methylierung von $(Cl_3Si)_2$–CH–CH_3. *Z. Anorg. Allgem. Chem.*, 322 (1963) 41–45 — retention data of 6 derivatives on silicone grease at 180–250°.

HILL, R. D. AND GESSER, H.: An investigation into the quantitative gas chromatographic analysis of metal chelates using a hydrogen-flame ionization detector. *J. Gas Chromatog.*, 1, No. 10 (1963) 11–14 — separation of Be, Al, Cu, Cr and Fe as acetonylacetonates, trifluoro- and hexafluoroacetonylacetonates by PTGC 85–130°.

ORTEGA, I. AND CYRUS, J. D.: Chromatographic liquid leak detection. *Anal. Chem.*, 35 (1963) 1649–1651 — volatile silicone fluid leaking from scaled containers.

ROSS, W. D.: Detection of metal chelates in gas liquid chromatography by electron capture. *Anal. Chem.*, 35 (1963) 1596–1598 — acetonylacetonates give higher electron affinity for Cr than for Al and higher for hexafluoro than trifluoro or non-fluoro derivatives.

SIEVERS, R. E., PONDER, B. W., MORRIS, M. L. AND MOSHIER, R. W.: Gas-phase chromatography of metal chelates of acetylacetone, trifluoroacetylacetone, and hexafluoroacetylacetone. *Inorg. Chem.*, 2 (1963) 693–698 — TFA complexes of Be, Al, In, Cr, Fe, Cu, Rh, Zr, Hf and geometrical isomers of $Cr(TFA)_3$.

29. INSECTICIDES AND OTHER PESTICIDES

COOK, J. W.: Assay of insecticides and herbicides in fats and oils. *J. Am. Oil Chemists' Soc.*, 40 (1963) 313–318 — GC is proposed for analysis.

31. PLASTICS AND THEIR INTERMEDIATES

ZULAICA, J. AND GUIOCHON, G.: Fast qualitative and quantitative microanalysis of plasticizers in plastics by gas liquid chromatography. *Anal. Chem.*, 35 (1963) 1724–1728 — SE-30 or poly-(neopentylglycol adipate) on glass beads at 200–240°.

32. PHARMACEUTICAL APPLICATIONS

SHAW, P. D.: Gas chromatography of trimethylsilyl derivatives of compounds related to chloramphenicol. *Anal. Chem.*, 35 (1963) 1580–1582 — 18 trimethylsilyl derivatives were separated in 13 peaks by means of TPGC at 110–260°.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

ABEL, K. AND DE SCHMERTZING, H.: Dual-chamber micro cross-section detector for permanent gas analysis. *Anal. Chem.*, 35 (1963) 1754–1756.

HYNEK, R. J. AND NELEN, J. A.: Gas chromatographic determination of nitrogen in refractory materials. *Anal. Chem.*, 35 (1963) 1655–1657 — nitrogen in gases from induction furnace combustion (1500° in oxygen) in AlN, BN, CrN, Si_3N_4, TiN, ZrN, AlN·SiC and B_6Si samples.

MIRZAYANOV, V. S., ZHUKHOVITSKIĬ, A. A., BEREZKIN, V. G. AND TURKEL'TAUB, N. M.: (Frontal displacement method of concentration of low adsorbed impurities). *Zavodsk. Lab.*, 29 (1963) 1166–1169 — 10^{-1} p.p.m. CO, O_2, N_2, CH_4 and C_2H_6 in ethylene.

RUBIN, I. B.: Modification of Perkin-Elmer vapor fractometer. *U.S. Gov. Res. Rep.*, ORNL-3397 UC-4 (1963) 39–41 — combined silica gel and molecular sieve (5 A) columns for simultaneous separation of CO_2 with permanent gases.

33b. Volatile inorganic substances

ARAKI, S., KATOH, T. AND ATOBE, T.: Gas chromatography of reactive inorganic gases and vapors. *Japan Analyst*, 12 (1963) 450–457 (English summary, p. 457) — retention data of nitrogen oxides, halogens, hydrogen halides on Kel-F on Teflon powder.

NELSON, D. F. AND KIRK, P. L.: Preparation of a compound which may be hydrogen isocyanide. *J. Chromatog.*, 12 (1963) 167–170 — identification of HNC as negative signal from ^{90}Sr detector.

ROCHEFORD, O.: Analyse des composés fluorés corrosifs par chromatographie gazeuse. Contrôle du fluor libre. *Anal. Chim. Acta*, 29 (1963) 350–357 — F_2 in UF_6, HF and permanent gases or *vice versa*.

WALLIS, J. M.: Chromatograph finds ammonia water content. *Oil Gas J.*, 61, No. 33 (1963) 108 — H_2O and NH_3 at 175° with accuracy of 0.02 wt. % (content 0.47 wt. %).

34. RADIOACTIVE COMPOUNDS

ADLOFF, J. P. AND GUEGUENIAT, P.: Application du détecteur à capture électronique en chimie des radiations. *J. Chromatog.*, 12 (1963) 96–98 — alkyl iodides from γ-irradiation of heptane in presence of iodine.

DRAWERT, F. AND BACHMANN, O.: Neuere Methoden zur Trennung und kontinuierlichen Messung von ^{14}C-Verbindungen in der Gasphase. *Angew. Chem.*, 75 (1963) 717–722.

DUTTON, H. J.: Reaction of unsaturated organic compounds with tritium gas. *Advances in Tracer Methodology*, Vol. 1, Plenum Press, New York, 1963, pp. 18–27 — labelled fatty acids on Resoflex 296 at 206°.

HORTON, A. D. AND MEYER, A. S.: Trapping and gas chromatographic determination of krypton, xenon, and carbon tetrafluoride. *U.S. Gov. Res. Rep.*, ORNL-3397 UC-4 (1963) 48–50 — molecular sieve 13X at 50°.

POPJÁK, G, LOWE, A. E. AND MOORE, D.: Simultaneous measurement of ^{14}C and ^{3}H during gas–liquid chromatography. *Advances in Tracer Methodology*, Vol. 1, Plenum Press, New York, 1963, pp. 127–146.

TOLBERT, B. M.: Tritium measurement using ionization chambers. *Advances in Tracer Methodology*, Vol. 1, Plenum Press, New York, 1963, pp. 167–177.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

ATTRILL, J. E.: Study of gas formation in inorganic moderates. *U.S. Gov. Res. Rep.*, ORNL-3397 UC-4 (1963) 39.

CHOY, T. K., QUATTRONE, Jr., J. J. AND ALICINO, N. J.: A gas chromatographic method for the determination of the antioxidants BHA, BHT and ethoxyquin in aqueous and in hydrocarbon soluble samples. *J. Chromatog.*, 12 (1963) 171–177 — in p.p.m. concentrations on SE-30.

FIRESTONE, D., IBRAHIM, W. AND HORWITZ, W.: Chick edema factor. III. Application of microcoulometric gas chromatography to detection of chick edema factor in fats or fatty acids. *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 384–396 — retention data of chlorinated pesticides and fatty acids on silicone grease at 248°.

INGLE, Jr., J. D.: Study of the volatile constituents of organic oils by gas chromatography. *J. Gas Chromatog.*, 1, No. 8 (1963) 34–36 — on EGS by TPGC at 100–170°.

SCHOLZ, R. G.: Relative retention data. *J. Gas Chromatog.*, 1, No. 10 (1963) 31 — retention times of 13 hydrocarbons and 46 oxygenated derivatives on Carbowax 20M at 125°.

A THEORY OF GEL FILTRATION AND ITS EXPERIMENTAL VERIFICATION

TORVARD C. LAURENT AND JOHAN KILLANDER

Department of Medical Chemistry and Department of Clinical Chemistry at the University Hospital, University of Uppsala, Uppsala (Sweden)

(Received December 17th, 1963)

Gel filtration the well known method of separating substances differing in molecular size, by passing a solution through a bed of gel grains, was described by PORATH AND FLODIN[1]. These authors used mainly cross-linked dextran gels (Sephadex, AB Pharmacia, Uppsala), but other polymer gels, such as those prepared from agar, starch, polyacrylamide, polyvinyl alcohol, etc., are also capable of producing similar separations. A review of the technique has recently been published by PORATH[2]. Although gel filtration is not strictly a process of filtration in the classical sense, the term will be used in the following, because of its common acceptance.

Many investigations have shown that a substance having large molecules generally passes through the gel bed faster than one having small molecules, provided that the concentration of polymer in the gel grains lies within a suitable range. Deviations from this pattern have only been observed in cases where it seemed reasonable to believe that an adsorption of the substance to the polymer had occurred[2-4]. The possibility of adsorption during gel filtration will not be discussed here, only the process that leads to a separation of molecules according to size will be treated.

Essentially two explanations for the gel filtrations process have appeared. STEERE AND ACKERS[5] have explained it as a decreased diffusion rate of the substance in the gel grains, and LATHE AND RUTHVEN[6], PORATH[7] and PEDERSEN[8] have discussed it in terms of an exclusion of the substance from the gel. Both mechanisms certainly operate in gel filtration. It has been shown that the diffusion rate of a macromolecule is decreased in both polysaccharide gels[9] and polysaccharide solutions[10] and that this decrease is a function of the size of the macromolecule. Furthermore it has been shown, both with the aid of equilibrium dialysis[11] with osmometric determinations[12] and with solubility studies[13,14], that polysaccharides such as hyaluronic acid and dextran exclude a certain volume of solvent for other solute macromolecules.

THEORY

It has been demonstrated that the position at which a substance is eluted in a gel chromatogram does not vary appreciably with the flow rate of the eluent[1,4]. This indicates that it is the volume available for a substance in the gel grains (total volume of gel phase minus excluded volume), and not the decreased diffusion rate, that determines the position of the substance in the chromatogram, for in the latter case the

elution volume would be strongly dependent on the flow rate. In the following treatment it will therefore be tacitly assumed that the volume in the gel grains available for a particular substance will determine its position in the gel chromatogram.

The relation between the elution volume and the available volume in the gel grains

A gel bed can be regarded as consisting of two phases, one gel phase with the volume V_x and one liquid phase with the volume V_0 (void volume). The resistance towards flow of water in the gel phase is very high (compare *e.g.* the high flow resistance in polyacrylamide gels[15]) and therefore it can be assumed that all water flow takes place in the liquid phase (see Fig. 1).

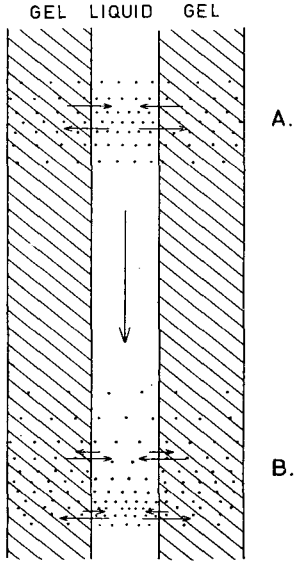


Fig. 1. Schematic diagram showing the most important mechanisms operating when a solute (dotted area) moves along a bed of gel grains. The diffusion of the solute (horizontal arrows) distributes it between the liquid phase (void volume) and the available volume in the gel phase. The flow of the liquid phase (vertical arrow) transports that fraction of the solute that at each moment is outside the gel grains. The lower density of solute in the gel phase indicates that only part of the gel phase is available for the solute. A. The ideal case of diffusion equilibrium between the gel and the liquid phases. B. The non-ideal condition, when the solute zone in the liquid phase moves ahead of that in the gel phase.

In the ideal case, a substance introduced in the system will equilibrate instantly between the liquid phase and the gel phase (Fig. 1A). Only a certain fraction, K_{av}, of the gel phase is, however, available for the substance, which means that the total available volume in the gel grains is $K_{av} \times V_x$. If a solute is introduced on the top of the gel bed and if the gel completely excludes this solute, it will be eluted after a volume, V_e, which is equal to the void volume. If, however, a volume in the gel phase is available for the solute, only that part that is in the liquid phase at each moment will move down the bed. The average speed of all solute molecules will be equal to the flow rate times that fraction of the molecules that in each instance is present in the liquid phase. In the ideal case, in which an instant equilibrium between the gel phase

and the liquid phase is assumed, the fraction of solute molecules that moves at each moment will be:

$$\frac{V_0}{V_0 + K_{av} \times V_x} \tag{1}$$

The elution volume of the substance will therefore be $(V_0 + K_{av} \times V_x)/V_0$ times larger than that of a substance that is completely excluded from the gel phase. The equation for the elution volume of the substance will thus be:

$$V_e = V_0 + K_{av} \times V_x \tag{2}$$

If the total volume of the gel bed, V_t ($V_t = V_0 + V_x$), is introduced, eqn. (2) can be rearranged to:

$$K_{av} = \frac{V_e - V_0}{V_x} = \frac{V_e - V_0}{V_t - V_0} \tag{3}$$

According to eqn. (3), it is possible to calculate K_{av} (*i.e.*, the fraction of the volume of the gel that is available for the substance) from the elution volume of the substance, the total volume of the gel bed and the void volume.

Influence on the gel filtration process of flow rate and the rate with which equilibrium between liquid phase and gel phase is attained

In the preceding section, it was assumed that equilibrium between liquid and gel will take place instantly. This is an ideal condition, which cannot be attained in practice. A study of the influence that a finite diffusion rate of the solute into the gel may have on the resolution in gel filtration is at present in progress in our laboratories and no quantitative presentation will be given here. Only a short qualitative description will follow (Fig. 1B).

The rate at which diffusion equilibrium is reached is governed by the size of the gel grains and the diffusion rate of the solute in the gel. The latter is a complicated function of the free diffusion rate, the molecular size of the solute and the concentration and structure of the gel substance[9,10].

When a solute passes through the gel bed with a finite flow rate, the diffusion between the liquid phase and the gel phase in front of the solute zone will not be sufficiently rapid to lead to an equilibrium. The result is a higher concentration in the liquid phase than in the available gel space. On the other hand, in the rear part of the solute zone, the concentration in the available space in the gel phase will be higher than in the liquid phase. The result can be regarded as if the solute zone in the liquid phase moves ahead of the solute zone in the gel phase. The effect will be more pronounced the higher the flow rate and give rise to a broadening and asymmetry of the solute band. The elution volume will, however, change relatively little, as will be shown in a subsequent paper.

Diffusion along the gel bed

If diffusion takes place along the gel bed, this will cause further dilution of the solute and broadening of the band, but the elution volume will not change.

Calculation of the available volume in a polymer network

In order to calculate the available volume in a gel, it is necessary to design a physical model of the gel network. The simplest model will be obtained by assuming that the dextran chains are straight rigid rods, which are infinitely long and distributed at random in the gel. OGSTON[16] has calculated the available volume for spherical particles in such a system. He arrives at the following formula:

$$K_{av} = \exp\left[-\pi L(r_s + r_r)^2\right] \tag{4}$$

where L is the concentration of rods in the solution, expressed as cm rod per cm^3, r_s is the radius of the spherical particles and r_r the radius of the rod.

This picture will of necessity be an approximation. The dextran molecule is branched, there are cross-linkages and a certain degree of rotation can take place at the glucosidic bonds. Also micro-heterogeneities may occur in the gel. It is, however, reasonable to make the assumption that these deviations from a linear straight polyglucose chain will essentially lead to a shortening and thickening of the chain. The molecule is still considered to have a rigid rodlike character.

The theory can then be summarized as follows: the volume available for a substance in the gel determines its position in the elution diagram; this volume can be calculated from the assumed physical model of the gel structure.

EXPERIMENTAL

Materials

Human fibrinogen, about 95 % coagulable, was kindly supplied by AB Kabi, Stockholm. Ribonuclease from bovine pancreas, crystallized five times (lot No. 32143), and cytochrome *c* from equine heart (assay 71 %, lot No. 32117) were obtained from California Corporation for Biochemical Research, Los Angeles, Calif. Horse myoglobin I was kindly supplied by Dr. A. EHRENBERG, Stockholm and violet dextran (molecular weight $\sim 2 \cdot 10^6$) by AB Pharmacia, Uppsala.

Methods

Gel filtration was performed as described in detail elsewhere[17]. All experiments were made on Sephadex G-200 with a water regain of 19.9 g/g gel, dry mesh 200–270 (U.S. sieve series). The columns had a diameter of 4.2 cm and lengths of 70.5 or 71.7 cm. The samples were applied in 14 ml volume. As buffer, 0.1 *M* Tris–HCl, pH 8.0 was used, containing 0.5 *M* sodium chloride and 0.02 % sodium azide. The transmission at 254 mμ was continuously registered in a Uvicord absorptiometer (LKB-Produkter, Stockholm). The substances were also determined in the collected fractions by spectrophotometry at their absorption maxima, and in the case of fibrinogen also as coagulable protein.

Results

The results are presented in Fig. 2. The ratio between elution volume and total volume is plotted on the abscissa. It can be assumed that the elution volume of violet dextran is equal to the void volume of the bed.

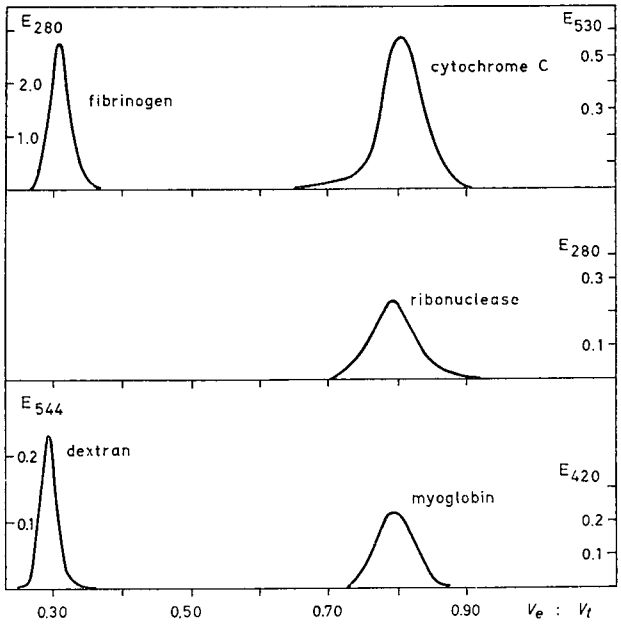


Fig. 2. Gel filtration of four proteins and violet dextran on Sephadex G-200.

COMPARISON BETWEEN THEORY AND EXPERIMENTS

The data presented above as well as data on the following substances taken from the literature have been used to test the hypothesis: oligosaccharides from cellulose[18] and dextran fractions[19] chromatographed on Sephadex G-25; dextran fractions[19] chromatographed on Sephadex G-50; dextran fractions[19] and proteins[20,21] chromatographed on Sephadex G-75; proteins[20,21] chromatographed on Sephadex G-100; and proteins[17,21,22] chromatographed on Sephadex G-200.

Calculation of K_{av}

FLODIN AND ASPBERG[18] and GRANATH AND FLODIN[19] have published data on void volumes, total volumes and elution volumes obtained in their experiments. These data were used to calculated K_{av} according to eqn. (3). It should be pointed out that K_{av} is not the same as K_d listed by GRANATH AND FLODIN, since K_d includes a correction term for the volume occupied by the dextran chains in the gel. Only those dextran fractions, collected by GRANATH AND FLODIN, that contained at least half as much material as the largest fraction, have been used for the calculations.

ANDREWS[20] has listed his data as migration rates of the substances on the gel columns divided by the migration rate of serum albumin. The elution volumes of albumin were taken from Fig. 1 in ANDREWS' paper. The total volume of each column was 226 ml and the elution volume of India ink and thyroglobulin has been assumed to be equal to the void volume. The elution volumes of four enzymes were estimated from data in Table IV and Fig. 1 in ANDREWS' paper. K_{av} has been calculated from these data. DETERMANN AND GELOTTE[21] present their data as K_d values. These

TABLE I

ESTIMATED MOLECULAR RADII AND K_{av} VALUES FOR SUBSTANCES STUDIED BY ANDREWS[20], DETERMANN AND GELOTTE[21], KILLANDER[17,22] AND THE PRESENT AUTHORS

Substance	$D_{20,w} \times 10^7$	*Ref.*	$r_s \times 10^8$	K_{av}					
				G-75		*G-100*		*G-200*	
				ANDREWS	DETERMANN AND GELOTTE	ANDREWS	DETERMANN AND GELOTTE	KILLANDER *et al.*[a]	DETERMANN AND GELOTTE
Sodium barbiturate			~ 4[b]					(0.94)	
Sucrose	41.6	24	5.1	0.88		0.94			
Vitamin B_{12}			~ 7.5[b]					(0.92)	
Cytochrome *c* (equine heart)	13.0	27, 28	16.4	0.43	0.38	0.59	0.66	0.72	
Myoglobin (horse)	10.3; 11.3	26, 28	20.7; 18.8	0.36		0.54		0.71	
Ribonuclease	11.1	29	19.2	0.43	0.33	0.58		0.70	0.74
Chymotrypsin	10.2	28	20.9		0.26		0.51		0.69
Trypsin	11.0	28	19.4		0.26		0.51		0.69
α-Lactalbumin (cow)	10.6	28, 30	20.1	0.39		0.56			
Chymotrypsinogen	9.5	28	22.4	0.27		0.45			
Soybean trypsin inhibitor	9.4[c]	31, 32	22.6	0.28		0.46			
Pepsin (monomer)	9.3	28	22.9		0.23				0.57
Cytochrome *c* (dimer)	9.1	27	23.4	0.25		0.42			
Ovalbumin	7.8	28	27.3	0.12		0.29	0.22		
Alkaline phosphatase (*E. coli*)	7.3[c]	33	29.2			0.17			
Peroxidase (horse radish)	7.05	34	30.2			0.27			
Hemoglobin, human	6.8	28	31.3	[d]	0.07	[d]	0.28[e]	0.49 (0.46)	0.50
Alkaline phosphatase (bovine intestine)	6.2[c]	35	34.6			0.08			
Serum albumin, human	5.9-6.1	36	36.1; 34.9	0.04		0.19	0.15	0.41 (0.37)	0.42
Heme-binding β-globulin, human	5.6[c]	37	38.0					0.36	
Transferrin, human	5.3; 5.9	38, 39	40.2; 36.1		0.00		0.10	0.37 (0.33)	0.31
Serum albumin (dimer)	4.9[c]	8	43.5			0.06			0.25
Alcohol dehydrogenase (yeast)	4.7	40	45.3			0.07			
Ceruloplasmin, human	4.7; 4.5; 3.8	41, 42	45.3; 47.3; 56.1					0.28 (0.24)	
Haptoglobin 1-1, human	4.7	43	45.3					(0.20)	
Phycocyanin	4.1	28	52.6						0.22
γ-Globulin (7-S), human	3.8	28	55.5	0.00	0.00	0.05	0.00	0.21 (0.19)	0.25
Haptoglobin 1-1–hemoglobin, human	3.4	43	62.6					(0.12)	
Fibrinogen, human	2.0	44	107		0.00		0.00	0.02	0.00

[a] Obtained on a lot of Sephadex G-200 with water regain 19.9. Figures within parentheses were obtained on lots with water regain 18.5–19.3.
[b] Approximate estimation from molecular weight, assuming the molecules to be spherical.
[c] Calculated with SVEDBERG's equation from molecular weight, $S_{20,w}$ and partial specific volume.
[d] ANDREWS reports that the elution volume for hemoglobin is dependent on concentration.
[e] The same value has been obtained by the present authors.

TABLE II

ESTIMATED MOLECULAR RADII AND K_{av} VALUES ON SEPHADEX G-25 FOR DEGRADATION PRODUCTS FROM CELLULOSE

Substance	*Estimated* $r_s \times 10^8$	K_{av}
Glucose	3.9	0.58
Cellobiose	5.1	0.52
Cellotriose	6.2	0.47
Cellotetraose	7.2	0.42
Cellopentaose	8.1	0.37
Cellohexaose	8.9	0.34

values, using 0.61 as the value of the partial specific volume of dextran[25], have been recalculated to K_{av}. Only those proteins for which reliable $D_{20,w}$ values were found have been listed.

The data of KILLANDER[17,22] and those presented above are expressed as ratios between elution volume and total volume. They have been converted to K_{av} using the elution volume of violet dextran or of human macroglobulins as the void volume The K_{av} values are listed in Tables I, II and III.

TABLE III

ESTIMATED MOLECULAR RADII AND K_{av} VALUES FOR DEXTRAN FRACTIONS OF DIFFERENT MOLECULAR WEIGHT

M_n	*Estimated* $r_s \times 10^8$	K_{av}
	Sephadex G-25	
1,230	9.5	0.34
1,320	9.9	0.29
1,610	11.0	0.24
2,240	12.9	0.19
2,630	14.0	0.14
3,570	16.2	0.09
4,550	18.2	0.04
	Sephadex G-50	
1,250	9.6	0.55
1,450	10.3	0.51
1,760	11.3	0.44
2,000	12.1	0.39
2,280	12.9	0.35
2,880	14.5	0.30
2,880	14.5	0.27
3,690	16.5	0.21
	Sephadex G-75	
1,350	10.0	0.71
1,790	11.5	0.63
2,270	12.9	0.55
2,690	14.1	0.50
3,180	15.3	0.45

Calculation of molecular radius (r_s)

The radius of the equivalent sphere was calculated from the diffusion constant, using STOKE's formula[23], to obtain a general measure of the molecular size of each substance.

Proteins. The diffusion constants ($D_{20,w}$) of the various proteins have been taken from the literature and are listed in Table I together with the calculated values of the radii. For some proteins, $D_{20,w}$ has been calculated from known molecular weights, $S_{20,w}$ and partial specific volumes using SVEDBERG's equation[23]. For a few proteins more than one value has been listed.

Carbohydrates. The diffusion constants for three monosaccharides, a disaccharide and a trisaccharide have been listed by LONGSWORTH[24]. GRANATH[25] has measured the diffusion constant of various dextran fractions (B512-Ph Dextran) with known number-average molecular weights. When the square root of the molecular weight was plotted *versus* the diffusion constant for these two sets of data, a linear relationship was obtained. This has been used for estimating the diffusion constants of the various cellulose and dextran fractions and their molecular radii (Tables II and III).

The radius of the dextran chain (r_r)

The radius of a straight polysaccharide chain is in the order of $2–3 \cdot 10^{-8}$ cm. However, the dextran in the gel is branched and flexible. The actual chain must therefore be assumed to be considerably thicker. A value for the radius of $7 \cdot 10^{-8}$ cm has been used throughout, because good agreement was then obtained between $\pi L r_r^2$ and the volume occupied by the dextran in the gel. For the estimation of L, see below.

Plotting of the data

The K_{av} values calculated from the data determined by ANDREWS on Sephadex G-75 and G-100, and by KILLANDER and the present authors on Sephadex G-200 are plotted in Figs. 3 and 4 *versus* the molecular radius. The curves are drawn according to eqn. (4), with a value for r_r of $7 \cdot 10^{-8}$ cm. The value of L was chosen for closest fit to the experimental values and is for G-75, $4.6 \cdot 10^{12}$; for G-100, $2.9 \cdot 10^{12}$; and for G-200, $1.6 \cdot 10^{12}$. The experimental values are in general close to the theoretical curves and the deviation can in most cases be explained by uncertainties in diffusion data and K_{av} values. The most notable discrepancies are shown by the alkaline phosphatases when chromatographed on G-100. They fall far below the theoretical curve. 7-S γ-globulin seems to fall above the line and so does ceruloplasmin. Sucrose and ovalbumin also deviate.

The points at r_s equal to zero are obtained by calculating the volume occupied by the dextran chains and subtracting this from the total volume of gel grains. The volume of dextran is calculated from its concentration and partial specific volume[25], 0.61.

The data given by DETERMANN AND GELOTTE are generally in agreement with the data presented in Figs. 3 and 4 (See Table I). There is, however, a larger scattering of their points, probably due to the fact that they have been obtained in two laboratories under different experimental conditions.

Fig. 5 shows the same kind of plot with the cellulose and dextran oligosaccharides chromatographed on Sephadex G-25, G-50 and G-75. The values of L chosen for the theoretical curves are $14.0 \cdot 10^{12}$, $8.2 \cdot 10^{12}$ and $4.8 \cdot 10^{12}$ respectively. The experi-

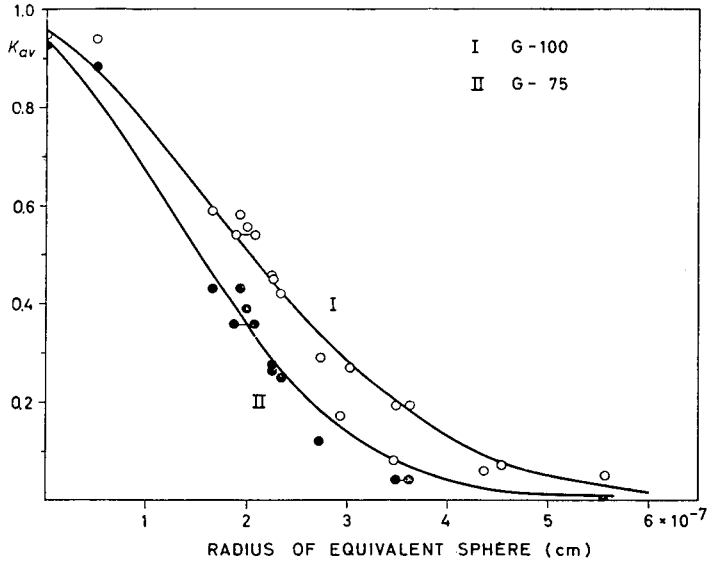


Fig. 3. K_{av} values calculated from the experimental data of ANDREWS plotted *versus* the radii of the equivalent spheres. The lines are drawn according to eqn. (4) using the values $2.9 \cdot 10^{12}$ (I) and $4.6 \cdot 10^{12}$ (II) for L. Points on the ordinate indicate calculated total volume of solvent in the gel grains.

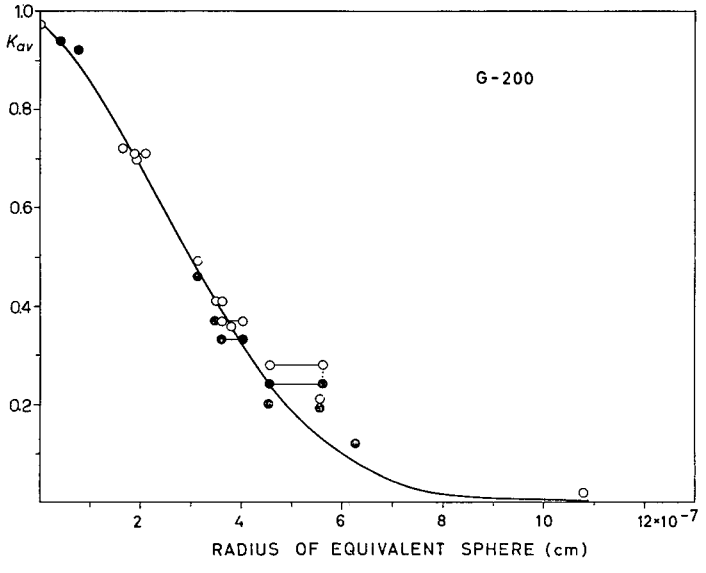


Fig. 4. K_{av} values calculated from the experimental data of KILLANDER and the present authors obtained on Sephadex G-200 plotted as in Fig. 3. A value of $1.6 \cdot 10^{12}$ for L is used. Open circles show experiments with a gel with water regain 19.9; filled circles represent experiments on gels with water regain 18.5–19.3.

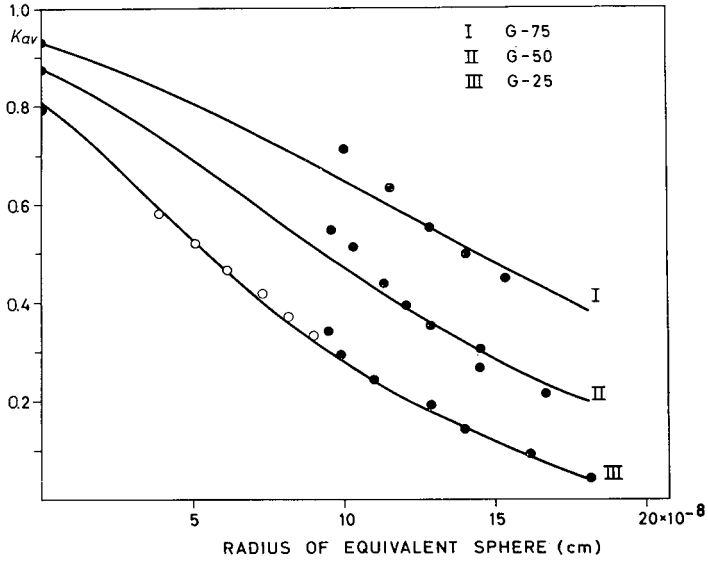


Fig. 5. The K_{av} values for degradation products from cellulose (○) and dextran (●) plotted as in Figs. 3 and 4. Lines are drawn according to eqn. (4) using the values $4.8 \cdot 10^{12}$ (I); $8.2 \cdot 10^{12}$ (II); and $14.0 \cdot 10^{12}$ (III) for L.

mental points fall somewhat above the lines for the small dextran molecules and below the lines for the larger.

Relation between L and the polymer concentration

The polymer concentration of the gels can be calculated from the water regain values (W_r) of the gels, using 0.61 for the partial specific volume of dextran[25]. The W_r value is equal to the number of ml of water held in the gel grains per gram of dextran. The values are, however, relatively uncertain due to difficulties in the determinations. The dextran concentrations can only be regarded as known within approximately 10 %.

The W_r values of the gels used by ANDREWS were 7.9 and 9.7, respectively (communication from AB Pharmacia) and those used by the present authors 19.9 and 18.5–19.3 respectively. The values obtained on the gel with $W_r = 19.9$, were used for fitting the curve in Fig. 4. FLODIN AND ASPBERG do not state the W_r of their gels. It is assumed to be the same as for the Sephadex G-25 used by GRANATH AND FLODIN. The latter authors used gels with W_r values of 2.3, 4.3 and 8.7 respectively.

Fig. 6 shows the relationship between L and the dextran concentration. An almost linear relationship is obtained in agreement with the hypothesis leading to eqn. (4).

From Fig. 6 it was found that in a dextran gel with the concentration 0.1 g/ml, L has a value of $4 \cdot 10^{12}$ cm/cm^3. If it is assumed that the length of a monosaccharide is $5 \cdot 10^{-8}$ cm, the total length of the dextran chains in 0.1 g of dextran was calculated to be $18.6 \cdot 10^{12}$ cm or approximately 4 to 5 times more than the experimental value. This ratio can be taken as an estimate of how much the dextran chain has been shortened by branching, flexibility in glucosidic linkages etc.

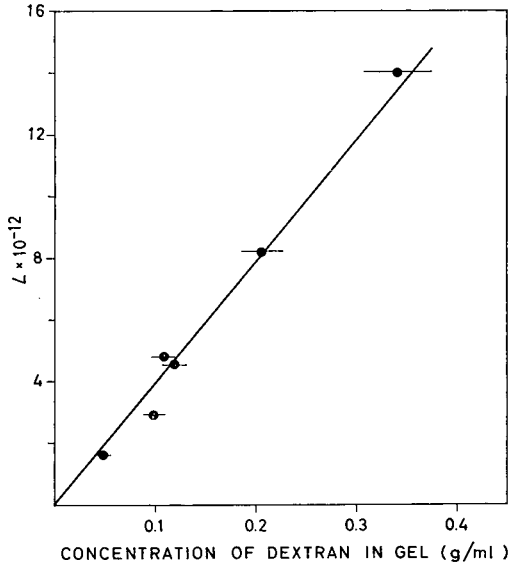


Fig. 6. The values of L used in Figs. 3, 4 and 5 plotted *versus* the concentration of dextran in the gel grains. Horizontal lines indicate $\pm$ 10% error in the calculated concentrations.

DISCUSSION

The present work was initiated in order to give a theoretical explanation of the well known observation that molecules of various sizes can be separated by chromatography on a bed of gel grains. It has been pointed out by some of the earlier authors that this effect may be due to an exclusion from the grains[6–8]. So far only PORATH[7] has tried to deduce a theoretical formula for the exclusion on the assumption that the free spaces in the gel grains are conical. He has tested his model using the dextran fractions obtained by gel filtration by GRANATH AND FLODIN[19]. PORATH gives an equation, which theoretically should only be valid for randomly coiled macromolecules and not for globular proteins. Unfortunately the test does not seem to be very sensitive, and DETERMANN AND GELOTTE[21] and ANDREWS[20] show that proteins give as good a fit as dextran fractions.

In the theory presented here, it is assumed that the gel network can be approximately treated as a three-dimensional network of randomly distributed straight fibers. The experiments using substances of a large range of molecular size and using a number of gels differing in polymer concentration show that if this model is used, most of the gel filtration data can be satisfactorily explained on the basis of an exclusion of molecules from part of the gel.

In some cases, however, there is a deviation from the expected values. Most deviations can be explained by the uncertainty in diffusion data and the approximation involved in calculating an equivalent radius from these data. A good example of the uncertainty in the values of the diffusion constants is given by ceruloplasmin, where available values in the literature disagree by as much as 20%. The diffusion constant of 7-S γ-globulin is also probably too low, since all 7-S γ-globulins studied by diffusion probably contained higher molecular weight aggregates[45]. This could explain

the deviation of γ-globulin to the right from the theoretical line in Fig. 4. The error in the determination of K_{av} is less than in that of the radius. Repeated determinations[17] on the same gel batch gave values varying only within $\pm$ 0.01.

In some instances, notably the alkaline phosphatases, the deviation is too large to be explained by errors in measurements. In this case, it can be explained by a type of aggregation of the protein, as the points fall below the theoretical line in Fig. 3. If any points should fall above the line, one has to assume a disaggregation or an adsorption of the substance to the gel matrix. Some results obtained by ANDREWS[20] are pertinent as examples of the latter. He showed that hemoglobin and β-lactoglobulin had elution volumes that were dependent on concentration and this was interpreted as indicative of a disaggregation. On the other hand this was not observed by KILLANDER[17] with hemoglobin in gel filtration or ultracentrifugal studies, and DETERMANN AND GELOTTE[21] did not report similar effects.

In a recently published report WHITAKER[46] gives data obtained by gel filtration on Sephadex G-75 and G-100. Uncertainties in reported volume have, however, made it difficult to calculate accurate K_{av} values. Rough calculations show, however, that most of his data correspond to those of earlier authors and agree closely with the theoretical curves of Fig. 3. This is also the case for hemoglobin and ovomucoid, two proteins which according to WHITAKER behaved anomalously on the columns.

The dextran fractions deviate from the theoretical functions, as can be seen in Fig. 5. This is, however, to be expected. GRANATH AND FLODIN[19] chromatographed a polydisperse dextran sample on dextran gels. They determined the molecular weights of fractions recovered at certain elution volumes. These fractions are, however, also polydisperse. A fraction eluted before the maximum of the peak is contaminated to a greater degree with low molecular weight than high molecular weight material. Conversely, a fraction which is eluted after the maximum contains proportionately more high molecular weight contaminating material. The radii calculated from the molecular weight values should therefore deviate in a way similar to what is the case in Fig. 5 from that of a monodisperse sample eluted at the same volume. This is also the reason why only the main fractions obtained by GRANATH AND FLODIN have been used for the calculations.

The observation that the exclusion effect determines the position in the gel chromatogram where a solute is eluted, opens up the possibility of treating the dynamics of gel filtration. In a subsequent contribution, we intend to show how a solute zone changes during the chromatographic process if the initial zone width, the flow rate and the rate at which the solute attains equilibrium between the liquid phase and the gel phase are known.

An important observation was made during this study. LAURENT[13,14] studied the solubilities of various proteins in the presence of dextran solutions. From the decrease in solubility the volume available for, *e.g.*, serum albumin, γ-globulin, cyanomethemoglobin and fibrinogen was determined. The values obtained in 4.9 % and 9.7 % (extrapolated) dextran of a mol. wt. of $5 \cdot 10^5$ are shown in Table IV. The K_{av} values from chromatography on Sephadex G-200 and G-100, which contain these concentrations of polymer in the gel grains, are also given in this table. In the case of gel filtration, the value of oxyhemoglobin is given instead of that of cyanomethemoglobin. Taking into account the experimental errors in the various determinations, there is good agreement between the available volumes in solution and in the gel,

TABLE IV

COMPARISON BETWEEN AVAILABLE VOLUMES OF FOUR PROTEINS IN DEXTRAN SOLUTIONS AND DEXTRAN GELS

Protein	*Available volume in*			
	4.9 % dextran		*9.7 % dextran*	
	solution	*gel*	*solution*	*gel*
Cyanomethemoglobin	0.42	—	0.25	—
Oxyhemoglobin	—	0.49	—	0.28
Serum albumin	0.43	0.41	0.19	0.19
γ-Globulin	0.26	0.21	—	—
Fibrinogen	0.11	0.02	—	—

with the exception of fibrinogen. In the latter case it should be noted that the molecular weight of the soluble dextran and the fibrinogen are of the same order and that the error in the determination of very low K_{av} values is larger than for higher values.

The results indicate that the cross-linking does not essentially change the exclusion properties of a certain concentration of dextran, at least not for moderately large proteins.

It is very probable that the unequal partition of macromolecules between two polymer phases, which has been demonstrated by ALBERTSSON[47] can partly be explained in terms of unequal exclusion properties of the two phases.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. GELOTTE AND ANDREWS for providing copies of their manuscripts before publication, and to AB Pharmacia, Uppsala, for gifts of Sephadex and violet dextran. The investigation was supported by the Swedish Medical Research Council and the Swedisch Cancer Society.

SUMMARY

The separation by gel filtration of molecules varying in size is explained as a steric exclusion of solutes from the gel phase.

The volume available for a solute in the gel phase can be determined from the elution volume, the void volume and the total volume of the gel column. It has been calculated for a number of proteins and dextran fractions and for various dextran gels from data given in the literature as well as from some new data. The values were used to test the hypothesis that the exclusion takes place from a three-dimensional random network of straight polymer fibers distributed in the gel. The experimental data were found to verify the hypothesis.

The experimentally determined available volumes in the gel phase for three proteins are approximately the same as the available volumes in dextran solutions, having the same polymer concentrations as the gels. Therefore there seems to be no essential difference between the exclusion phenomenon in a polymer gel and in a polymer solution.

REFERENCES

1 J. PORATH AND P. FLODIN, *Nature*, 183 (1959) 1657.
2 J. PORATH, *Advan. Protein Chem.*, 17 (1962) 209.
3 B. GELOTTE, *J. Chromatog.*, 3 (1960) 330.
4 P. FLODIN, *Dextran Gels and Their Applications in Gel Filtration*, AB Pharmacia, Uppsala, 1962.
5 R. L. STEERE AND G. K. ACKERS, *Nature*, 196 (1962) 475.
6 G. H. LATHE AND C. R. J. RUTHVEN, *Biochem. J.*, 62 (1956) 665.
7 J. PORATH, *Pure Appl. Chem.*, 6 (1963) 233.
8 K. O. PEDERSEN, *Arch. Biochem. Biophys.*, Suppl. 1 (1962) 157.
9 G. K. ACKERS AND R. L. STEERE, *Biochim. Biophys. Acta*, 59 (1962) 137.
10 T. C. LAURENT, I. BJÖRK, A. PIETRUSZKIEWICZ AND H. PERSSON, *Biochim. Biophys. Acta*, 78 (1963) 351.
11 A. G. OGSTON AND C. F. PHELPS, *Biochem. J.*, 78 (1961) 827.
12 T. C. LAURENT AND A. G. OGSTON, *Biochem. J.*, 89 (1963) 249.
13 T. C. LAURENT, *Biochem. J.*, 89 (1963) 253.
14 T. C. LAURENT, *Acta Chem. Scand.*, in the press.
15 M. L. WHITE, *J. Phys. Chem.*, 64 (1960) 1563.
16 A. G. OGSTON, *Trans. Faraday Soc.*, 54 (1958) 1754.
17 J. KILLANDER, *Biochim. Biophys. Acta*, 93 (1964) 1.
18 P. FLODIN AND K. ASPBERG, in T. W. GOODWIN AND O. LINDBERG (Editors), *Biological Structure and Function*, Vol. 1, Academic Press, New York, 1961, p. 345.
19 K. A. GRANATH AND P. FLODIN, *Makromol. Chem.*, 48 (1961) 160.
20 P. ANDREWS, *Biochem. J.*, in the press.
21 H. DETERMANN AND B. GELOTTE, *Biochemisches Taschenbuch*, in the press.
22 J. KILLANDER, unpublished.
23 E. J. COHN AND J. T. EDSALL, *Proteins, Amino Acids and Peptides*, Reinhold, New York, 1943, pp. 402 and 422.
24 L. G. LONGSWORTH, *J. Am. Chem. Soc.*, 74 (1952) 4155.
25 K. A. GRANATH, *J. Colloid Sci.*, 13 (1958) 308.
26 A. EHRENBERG, *Acta Chem. Scand.*, 11 (1957) 1257.
27 E. MARGOLIASH AND J. LUSTGARTEN, *J. Biol. Chem.*, 237 (1962) 3397.
28 J. T. EDSALL, in H. NEURATH AND K. BAILEY (Editors), *The Proteins*, Vol. 1 B, Academic Press, New York, 1953, p. 549.
29 A. GINSBURG, P. APPEL AND H. K. SCHACHMAN, *Arch. Biochem. Biophys.*, 65 (1956) 545.
30 W. G. GORDON AND W. F. SEMMETT, *J. Am. Chem. Soc.*, 75 (1953) 328.
31 E. SHEPPARD AND A. D. MCLAREN, *J. Am. Chem. Soc.*, 75 (1953) 2587.
32 Y. V. WU AND H. A. SCHERAGA, *Biochemistry*, 1 (1962) 698.
33 A. GAREN AND C. LEVINTHAL, *Biochim. Biophys. Acta*, 38 (1960) 470.
34 R. CECIL AND A. G. OGSTON, *Biochem. J.*, 49 (1951) 105.
35 L. ENGSTRÖM, *Biochim. Biophys. Acta*, 52 (1961) 36.
36 J. T. YANG, *Advan. Protein Chem.*, 16 (1961) 323.
37 H. E. SCHULTZE, K. HEIDE AND H. HAUPT, *Naturwiss.*, 48 (1961) 696.
38 T. E. THOMPSON, cited by L. J. GOSTING, *Advan. Protein Chem.*, 11 (1956) 429.
39 G. BRÄSE, *J. Prakt. Chem.*, 13 (1961) 211.
40 J. E. HAYES, JR. AND S. F. VELICK, *J. Biol. Chem.*, 207 (1954) 225.
41 B. E. SANDERS, O. P. MILLER AND M. N. RICHARD, *Arch. Biochem. Biophys.*, 84 (1959) 60.
42 C. B. KASPER AND H. F. DEUTSCH, *J. Biol. Chem.*, 238 (1963) 2325.
43 G. HERMANN-BOUSSIER, *Les Haptoglobines Humaines*, Imprimerie R. Foulon, Paris, 1960.
44 H. A. SCHERAGA AND M. LASKOWSKI, JR., *Advan. Protein Chem.*, 12 (1957) 1.
45 R. A. PHELPS AND F. W. PUTNAM, in F. W. PUTNAM (Editor), *The Plasma Proteins*, Vol. 1, Academic Press, New York, 1960, p. 143.
46 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
47 P. Å. ALBERTSSON, *Partition of Cell Particles and Macromolecules*, John Wiley & Sons, New York, 1960.

BEITRAG ZUR GASCHROMATOGRAPHISCHEN ANALYSE DES LAVENDELÖLS

J. KOLŠEK UND M. MATIČIČ
Chemisches Institut "Boris Kidrič", Ljubljana (Jugoslawien)

(Eingegangen den 8. August 1963)

PROBLEMSTELLUNG

Lavendelöl, eines der populärsten ätherischen Öle, wird unter anderem auch in unserem Lande — besonders in Dalmatien — in beträchtlicher Menge gewonnen. Bekanntlich hängt die Zusammensetzung dieses ätherischen Öles in starkem Masse von der Abart der Pflanze, aus welcher das Öl isoliert wird, ab, wobei auch die klimatischen Bedingungen, die Beschaffenheit des Bodens u.a. einen merklichen Einfluss ausüben. Das Ziel unserer Arbeit war, die Zusammensetzung unseres einheimischen Öles auf gaschromatographischem Wege zu ermitteln und die Qualität verschiedener Muster dieses Öles untereinander als auch mit der Qualität französischer Öle zu vergleichen.

Mit gaschromatographischer Ermittlung der Zusammensetzung des Lavendelöls haben sich schon verschiedene Autoren befasst. Die meisten Untersuchungen führten NAVES und Mitarbeiter durch, die in diesem Öl verschiedene Terpene nachgewiesen haben, wobei sie Reoplex, Siliconfett und Squalan als stationäre Phase verwendeten[1-4]. STADLER und Mitarbeiter haben vor allem die im Lavandinöl enthaltenen Ketone untersucht. Diese wurden durch Behandlung mit Girard-Reagens P aus dem Öl isoliert und auf gaschromatographischem Wege auf polaren und unpolaren Phasen getrennt und näher identifiziert[5]. Als polare Phase diente Emulphor O, als nicht polare Phase dagegen Apiezon L. Auf Apiezon L haben dieselben Autoren weiterhin die Terpenalkohole aus französischem Lavendelöl untersucht[6]. Unterschiede in der Zusammensetzung des ätherischen Öls des echten Lavendels und des Lavandins wurden auf gaschromatographischem Wege durch NAVES ermittelt[7]. Auf der stationären Phase auf Siliconöl-Basis identifizierte er 8 Komponenten, die in einzelnen Ölsorten in verschiedenen Konzentrationen enthalten sind. In gleicher Weise bestimmte NAVES mittels gaschromatographischer Analyse an Squalan den Gehalt des Borneols im Lavendelöl[8]. Hierbei wurde festgestellt, dass ätherisches Öl aus echtem Lavendel kein Bornylacetat und nur geringe Mengen an Campher enthält. Zur gaschromatographischen Trennung von Linalool, Linalylacetat und Campher schlug BRODERICK ein Gemisch von Quadrol und Saib vor[9], dagegen wurde zu diesem Zweck seitens anderer Autoren Polyäthylenglykol als stationäre Phase verwendet[10]. Mit vergleichenden Untersuchungen verschiedener Muster des Lavandinöls und Lavendelöls befasste sich ausserdem FENAROLI[11].

Einer der wichtigsten Inhaltsstoffe des Lavendelöls ist Linalylacetat, das stets von Linalool und Campher begleitet wird. Durch verschiedenen mengenmässigen Gehalt vor allem dieser Komponenten unterscheiden sich verschiedene Sorten des

Öles, nämlich das ätherische Öl des echten Lavendels, des Lavandins und das Spiköl. Deswegen ist eine einwandfreie Trennung dieser Komponenten unter sich eine der wichtigsten Aufgaben, vor die man bei der gaschromatographischen Untersuchung des Lavendelöls gestellt ist. Die Schwierigkeiten, die man bei dieser Trennung begegnet, wurden schon durch BRODERICK[9] beschrieben, der zur Trennung erwähnter Komponenten eine geeignete Phase gefunden hat. Da wir jedoch nach diesen Angaben keine zufriedenstellende Resultate erzielen konnten, waren wir gezwungen, noch eine weitere Anzahl stationärer Phasen zu erproben, um eine gute Trennung von Linalylacetat, Linalool und Campher zu erreichen. Eine stationäre Phase mit sehr hoher Trennleistung war noch besonders erwünscht, da wir die Trennungen mit dem Pye-Gaschromatographen durchführen wollten, bei welchem die Länge der Standardsäule nur etwa 120 cm beträgt. Im ersten Teil unserer Arbeit führten wir demnach nur Versuche durch, bei welchen wir die Wanderungsgeschwindigkeiten von Linalylacetat, Linalool und Campher an verschiedenen Phasen ermittelten und die Auftrennung dieser Komponenten im Gemisch studierten. Nachdem wir in Castorwax eine hervorragende Phase fanden, die alle drei genannten Komponenten untereinander vollkommen aufzutrennen vermag und ausserdem eine gute Auftrennung der meisten übrigen Inhaltsstoffe des Lavendelöls herbeiführt, führten wir eine quantitative Vergleichsanalyse verschiedener Muster einheimischer Lavandinöle und einiger französischer Öle durch.

EXPERIMENTELLER TEIL

Apparatives

Zur Durchführung gaschromatographischer Analysen stand uns ein Gaschromatograph der Firma Pye (Cambridge, England) zur Verfügung. Die Aufdeckung der Substanzen erfolgt bei diesem Gaschromatographen mit Hilfe eines Ionisationsdetektors nach Lovelock, wobei als Trägergas Argon verwendet wird.

Säulen

Sämtliche Trennungen wurden an Standardsäulen von etwa 120 cm effektiver Länge und mit einem inneren Durchmesser von etwa 6 mm durchgeführt.

Trägersubstanz

Zur Bereitung stationärer Phasen für die Säulenfüllung diente säuregewaschenes Embacel (May & Baker) mit einer Korngrösse von 0.15–0.25 mm (60–100 mesh). Die Angaben über die Zusammensetzung der verwendeten stationären Phasen sowie über andere Arbeitsbedingungen sind aus der Tabelle I ersichtlich.

Standardsubstanzen

Die Muster von Reinsubstanzen, mit welchen die Trennleistung einzelner stationärer Phasen studiert wurde und an Hand welcher uns die Identifizierung einiger Inhaltsstoffe des Lavendelöls möglich war, wurden uns freundlicherweise von den Firmen Dragoco (Holzminden/Weser) und Riedel de Haën (Hamburg) zur Verfügung gestellt.

Wegen der äusserst hohen Empfindlichkeit des Ionisationsdetektors nach Lovelock war es nötig, nur sehr geringe Mengen von Lavendelöl zu dosieren. Aus diesem Grunde haben wir das ätherische Öl jeweils in Form der Lösung auf die Säule aufgetragen. Als Lösungsmittel verwendeten wir hierbei Tetrachlorkohlenstoff.

TABELLE I

STATIONÄRE PHASEN UND ARBEITSBEDINGUNGEN

Organische Phase	*Quadrol–Saib (2:1)*	*Saib*	*Carbowax 20 M*	*Reoplex 400*	*Hyprose*	*Polyäthylenglykoladipat*	*Polypropylenglykol*	*Castorwax*
Provenienz	*W & Co**	*W & Co*	*W & Co*	*W & Co*	*W & Co*	*Pye*	*W & Co*	*W & Co*
Konzentration der org. Phase an Embacel (Gew. %)	20	20	20	20	20	20	20	15
Säulentemperatur (°C)	110	120	110	110	100	110	110	120
Trägergas Einlassdruck (atm.)	0.20	0.20	0.10	0.15	0.10	0.20	0.25	0.18
Empfindlichkeitsstufe	10	10	10	10	10	10	10	10
Detektorelektrodenspannung (V)	1250	1250	1250	1250	1250	1250	1250	1250
Papiervorschub (cm/h)	40	40	40	40	40	40	40	40

* Wilkens & Co., U.S.A.

Unter den in Tabelle I angeführten Arbeitsbedingungen haben wir zunächst an verschiedenen stationären Phasen die Wanderungsgeschwindigkeiten von Linalylacetat, Linalool und Campher bestimmt. Die Ergebnisse dieser Versuche sind aus der Tabelle II zu entnehmen. Nachdem die Reinsubstanzen vereinzelt chromatographiert wurden, liessen wir sie auch zusammen im Gemisch wandern um auf diese Weise noch einmal die Trennleistung der Phase zu überprüfen.

TABELLE II

RELATIVE RETENTIONSWERTE EINZELNER REINSUBSTANZEN (BEZOGEN AUF CAMPHER) AN VERSCHIEDENEN STATIONÄREN PHASEN

(Arbeitsbedingungen, siehe Tabelle I)

Stationäre Phase	R_F		
	Linalool	*Linalylacetat*	*Campher*
Quadrol–Saib	0.97	1.00	1.00
Saib	0.90	1.32	1.00
Carbowax 20 M	0.97	1.01	1.00
Reoplex 400	0.96	1.04	1.00
Hyprose	0.77	1.03	1.00
Polyäthylenglykoladipat	0.88	0.99	1.00
Polypropylenglykol	1.01	1.56	1.00
Castorwax	0.81	1.19	1.00

RESULTATE

1. *Stationäre Phase auf Basis von Quadrol–Saib-Gemisch*

Linalool ist einer der Inhaltsstoffe verschiedener ätherischer Öle. Als ungesättigter tertiärer Alkohol ist diese Verbindung sowohl in freier Form als auch in Form seiner Ester ziemlich unbeständig und muss deshalb vor Licht, Wärme und Einfluss der Säuren geschützt werden. BRODERICK[9] gibt an, dass die Mehrzahl der stationären Phasen zur Trennung von Linalool und Linalylacetat nicht geeignet ist, da sie eine

Zersetzung dieser Substanzen während der Chromatographie hervorrufen. Als eine geeignete Phase, die eine Auftrennung des Gemisches von Linalool, Linalylacetat und Campher ermöglicht, ohne dass hierbei eine Zersetzung der empfindlichen Komponenten eintritt, wurde von BRODERICK das Gemisch von Quadrol [Tetrakis-(2-hydroxypropyl)-äthylendiamin] und Saib (Sucrose-acetatisobutyrat) im Verhältnis 2:1 vorgeschlagen. Nach Angaben des Autors ruft Saib allein ebenfalls eine Zersetzung von Linalylacetat und Linalool hervor. Die Zersetzung kann jedoch verhindert werden, falls die Trägersubstanz vor der Imprägnation mit Saib mit einer Lauge behandelt wird. Unter diesen Bedingungen sollen jedoch Linalylacetat und Campher ohne Trennung in einem gemeinsamen Elutionsmaximum wandern. Quadrol allein soll hingegen dieses Substanzpaar auftrennen, andererseits aber bleiben Linalylacetat und Linalool ungetrennt. Auf Quadrol, das selbst eine Base ist, tritt keine Zersetzung des Linalools und seiner Ester ein. Durch Kombination beider Substanzen, d.h. an einem Quadrol–Saib-Gemisch soll also eine Auftrennung sämtlicher drei genannter Komponenten stattfinden.

Bei unserer Arbeit versuchten wir zunächst, laut Angaben von BRODERICK die Trennung von Linalylacetat, Linalool und Campher auf der stationären Phase auf Basis von Quadrol–Saib (2:1) zu erreichen. BRODERICK führte die Trennung dieser Komponenten auf einer etwa 3 m langen Säule bei einer Arbeitstemperatur von 136° durch. Da unsere Säule nur etwa 120 cm lang war, konnten wir die Arbeitstemperatur auf 110° reduzieren um dadurch die Gefahr einer Zersetzung noch weiter herabzusetzen. Leider trat unter diesen Bedingungen keine Auftrennung der genannten Komponenten ein, sie wanderten vielmehr in einem gemeinsamen Elutionsmaximum. Auch durch Ändern der Arbeitstemperatur konnten wir mit unserer Säule keine Auftrennung dieses Substanzgemisches erzielen, da die Differenzen der Wanderungsgeschwindigkeiten einzelner Komponenten zu gering sind.

2. Stationäre Phase auf Basis von Saib

Im Gegensatz zu den Feststellung von BRODERICK konnten wir an Saib allein eine relativ gute Auftrennung eines Gemisches von Linalool, Linalylacetat und Campher erreichen. Da jedoch die Elutionsmaxima einzelner Komponenten asymmetrisch geformt sind (Fig. 1), ist auch Saib als stationäre Phase zu diesem Zweck wenig brauchbar. An Saib wird Linalylacetat zuletzt eluiert, da Saib eine selektive stationäre Phase darstellt, auf welcher Ester gegenüber den Alkoholen stärker zurückgehalten werden.

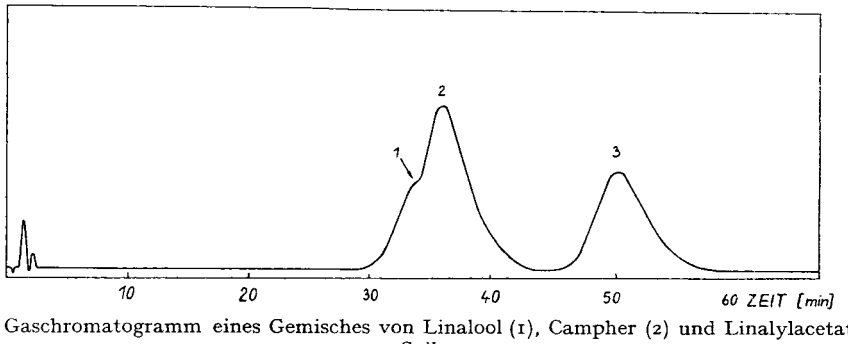


Fig. 1. Gaschromatogramm eines Gemisches von Linalool (1), Campher (2) und Linalylacetat (3) an Saib.

3. Stationäre Phase auf Basis von Carbowax 20M

Carbowax 20 M wurde schon öfters als stationäre Phase bei gaschromatographischen Untersuchungen der ätherischen Öle verwendet. Die Elutionsmaxima von Linalool, Linalylacetat und Campher sind an Carbowax 20 M praktisch völlig symmetrisch, die Differenzen der Wanderungsgeschwindigkeiten dieser Komponenten sind jedoch zu klein, um eine Auftrennung des Gemisches zu erreichen.

4. Stationäre Phase auf Basis von Reoplex 400

Ebenso wie auf Carbowax 20 M wanderte das Linalool–Linalylacetat–Campher-Gemisch auch auf Reoplex 400 ohne Auftrennung. Die Differenzen der Wanderungsgeschwindigkeiten einzelner Komponenten des Gemisches sind zwar auf dieser Phase etwas grösser als bei Carbowax 20 M, jedoch immer noch zu gering um auf einer 120 cm-Säule eine Trennung herbeizuführen.

5. Stationäre Phase auf Basis von Hyprose

Hyprose [Oktakis-(2-hydroxypropyl)-sucrose] wurde von CARTONI UND LIBERTI[12] als selektive Phase erkannt, da sie Alkohole gegenüber Aldehyden und Estern stärker zurückhält. Aus diesem Grunde haben wir versucht, ob wir an dieser Phase die gewünschte Trennung von Linalool, Linalylacetat und Campher erzielen könnten. Aus den in Tabelle II angegebenen Wanderungsgeschwindigkeiten einzelner Komponenten konnte schon der Beschluss erbracht werden, dass aus dem Gemisch dreier Komponenten Linalylacetat klar abgetrennt wird, Linalool und Campher dagegen ohne Trennung im gemeinsamen Elutionsmaximum wandern.

6. Stationäre Phase auf Basis von Polyäthylenglykoladipat

Auf dieser stationären Phase konnten wir ebenfalls nur eine Komponente des Gemisches abtrennen. Im Gegensatz zu Hyprose wanderte hier Linalool voran, dagegen blieben Linalylacetat und Campher wegen zu geringer Differenz ihrer Wanderungsgeschwindigkeiten ungetrennt.

7. Stationäre Phase auf Basis von Polypropylenglykol

Die Trennleistung von Polypropylenglykol ist sehr ähnlich derjenigen von Hyprose, da an diesen beiden Phasen nur die Abtrennung von Linalylacetat aus dem Linalool–Linalylacetat–Campher-Gemisch erzielt wird. Der Unterschied besteht lediglich darin, dass an Hyprose Linalylacetat vor Linalool und Campher eluiert wird, an Polypropylenglykol wandern dagegen Linalool und Campher vor Linalylacetat.

8. Stationäre Phase auf Basis von Castorwax

An allen bisher erwähnten stationären Phasen konnten wir im besten Falle die Abtrennung nur einer einzigen Komponente des Linalool–Linalylacetat–Campher-Gemisches erzielen. Wie schon anfangs erwähnt, konnten wir hingegen eine vollkommen einwandfreie Auftrennung dieses Gemisches in alle drei Komponenten an Castorwax erreichen. Castorwax ist ein durch Hydrierung von Rizinusöl gewonnenes Produkt und stellt chemisch Glyzerin-tri-12-hydroxystearat dar. Castorwax ist als stationäre Phase für die Gaschromatographie vor allem aus dem Grunde geeignet, da es Arbeitstemperaturen bis zu 200° verträgt. Diese Phase wurde erfolgreich zur gaschromatographischen Untersuchung der Aromastoffe der Vanille herangezogen[13].

Da verschiedene Komponenten aus ätherischen Ölen an Castorwax relativ hohe Retentionszeiten aufweisen, wählten wir beim Bereiten der Säulenfüllung eine niedrigere Konzentration (15%) der organischen Substanz an Embacel. Dadurch konnten wir die Arbeitstemperatur der Säule auf 120° senken ohne die Analysendauer unnötigerweise zu verlängern. Die unter diesen Arbeitsbedingungen verzeichneten Rückhaltezeiten von Linalool, Linalylacetat und Campher sind in Tabelle II angeführt, die Auftrennung des Gemisches dieser Komponenten zeigt Fig. 2.

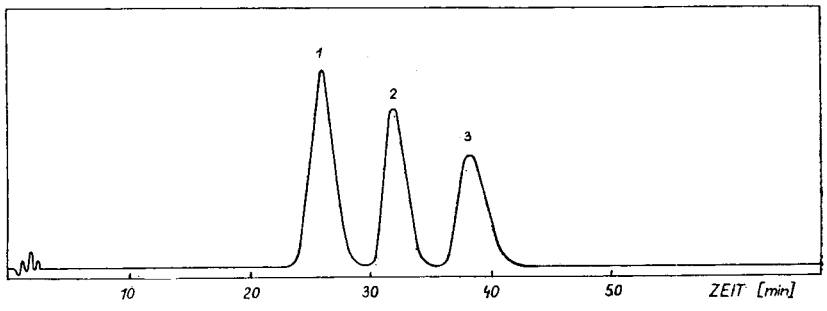


Fig. 2. Gaschromatogramm eines Gemisches von Linalool (1), Campher (2) und Linalylacetat (3) an Castorwax.

Die Elutionsmaxima sind an Castorwax praktisch völlig symmetrisch, eine Zersetzung trat weder bei Linalool, noch bei Linalylacetat ein. Die grosse Differenz der Wanderungsgeschwindigkeiten einzelner Komponenten ermöglicht eine einwandfreie Trennung auch an relativ kurzen Säulen.

Da demnach Castorwax eine hervorragende Phase zur Trennung des Gemisches von Linalool, Linalylacetat und Campher darstellt, haben wir seine Trennleistung noch gegenüber dem Lavendelöl ausprobiert. Auch hier stellten wir fest, dass von allen oben genannten stationären Phasen Castorwax die beste Auftrennung ermöglicht und die grösste Anzahl der Maxima liefert. Aus diesem Grunde verwendeten wir Castorwax auch zur Durchführung von Vergleichsanalysen, die die Qualität einiger Muster unserer einheimischen Lavendel- bzw. Lavandinöle zeigen sollten. Die Ergebnisse dieser Analysen sind in Tabelle III enthalten. Die nicht näher identifizierten Komponenten der Öle sind mit laufenden Buchstaben gekennzeichnet. In der Berechnung der Zusammensetzung der Öle wurden die Flächenprozente aus den Chromatogrammen einfach den Gewichtsprozenten der Komponenten gleichgesetzt. Um sämtliche Inhaltsstoffe der Öle erfassen zu können, führten wir die Auftrennung einmal bei kleinerer und das andere Mal bei grösserer Durchflussgeschwindigkeit des Trägergases (Einlassdruck 0.35 bzw. 0.17 atm.) durch. Ein typisches Gaschromatogramm des Lavendelöls zeigt Fig. 3.

Die Resultate dieser Analysen zeigen, dass die merklichsten Unterschiede in der Zusammensetzung des Lavandin- und Lavendelöls im Linalylacetatgehalt zu finden sind. Der Linalylacetatgehalt der untersuchten Muster der Lavendelöle schwankt zwischen 29.6 und 31.6%, bei Lavandinölen liegt er dagegen bei 3.7–7.2%. Ein weiterer Unterschied zeigt sich im Cineolgehalt. Lavandinöle enthalten in der Regel mehr Cineol (Eukalyptol) als Lavendelöle. In den untersuchten Mustern der Lavandinöle betrug der Cineolgehalt 5.5–9.2%, bei echtem Lavendelöl war er nur gering (0.5–1.3%). Schon NAVES stellte fest, dass sich Lavendelöl auch nach dem Gehalt

TABELLE III

VERGLEICHSANALYSE DER ZUSAMMENSETZUNG VERSCHIEDENER MUSTER DES EINHEIMISCHEN UND FRANZÖSISCHEN LAVENDELÖLS

1 = Lavandinöl aus Pitve (Insel Hvar); 2 = Lavandinöl aus Milna (Insel Hvar); 3 = Lavandinöl aus Dol (Insel Hvar); 4 = Lavandinöl aus Dalmatien; 5 = Lavandinöl aus Sučuraj (Insel Hvar); 6 = Lavandinöl aus Gdinj; 7 = Lavandinöl (Firma Gosad); 8 = Lavendelöl (nach Ph. J. II); 9 = Lavandinöl (Firma Kemijaimpex); 10 = Lavendelöl (Firma Etol); 11 = Lavendelöl aus Sučuraj (Insel Hvar); 12 = Lavendelöl aus Milna (Insel Brač); 13 = Lavendelöl, französisch; 14 = Lavendelöl Barreme; 15 = Lavendelöl Mont Blanc.

Komponent	*Prozentgehalt der Komponente im Ölmuster Nr.*														
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
A	—	—	—	0.5	0.2	0.1	—	—	0.3	—	—	—	—	—	—
α-Pinen	0.7	0.4	2.0	1.1	0.7	0.4	0.8	0.6	0.6	0.3	0.3	0.3	1.1	0.3	0.1
Camphen	0.4	0.3	1.0	0.5	0.3	0.2	0.4	0.4	0.3	0.3	0.3	0.3	0.5	0.3	0.2
β-Pinen	0.8	0.4	1.6	1.1	0.5	0.3	0.9	0.4	0.5	0.4	0.4	0.3	0.5	0.3	—
Δ^3-Caren	0.7	0.6	1.3	0.7	0.7	0.4	1.0	0.3	0.7	0.4	0.5	0.3	0.9	0.2	0.2
Dipenten	1.5	0.8	6.7	3.1	1.3	0.9	3.0	1.4	4.0	1.5	1.6	1.2	2.5	2.3	1.9
Cineol	7.6	9.2	7.7	8.2	6.7	5.5	6.1	4.9	4.6	0.5	1.0	1.3	9.5	1.5	3.0
B	—	—	—	—	—	—	—	—	—	0.9	—	—	5.4	—	—
C	0.7	0.5	1.5	0.7	0.8	0.5	1.0	0.7	0.7	0.6	1.0	0.6	0.6	0.6	0.4
D	—	0.1	—	—	0.2	0.2	0.1	1.4	—	1.2	1.7	1.5	0.3	1.2	1.2
E	0.3	—	—	—	0.2	1.7	0.2	—	—	—	—	0.4	—	—	—
F	0.3	1.2	—	—	0.7	0.4	0.4	4.5	—	0.5	0.9	1.7	—	—	4.9
G	0.1	1.2	—	—	0.7	0.3	0.5	6.3	—	0.4	0.7	1.4	0.7	—	4.6
Linalool	60.2	63.5	49.5	62.2	56.8	64.2	34.3	37.5	64.8	52.9	43.9	46.0	45.3	45.7	35.9
Campher	2.0	3.1	2.6	1.2	2.0	1.7	3.4	5.0	1.3	1.0	1.5	1.1	7.3	1.5	7.9
Linalylacetat	3.8	4.5	7.2	3.7	5.4	4.9	6.7	25.7	3.8	29.6	29.6	31.6	9.4	36.5	28.5
H	7.9	4.4	7.4	7.2	8.3	6.8	16.4	2.3	8.8	2.7	4.6	3.2	2.5	2.4	1.2
Isoborneol	0.8	0.7	1.3	0.5	0.9	0.8	1.5	1.6	0.7	3.3	4.8	3.8	1.0	2.7	1.8
Bornylacetat Isobornylacetat Borneol	8.6	7.0	7.7	7.5	11.2	7.9	19.9	1.7	8.0	1.2	2.1	1.7	2.7	1.6	2.5
I	—	—	—	—	—	—	—	1.1	—	—	—	—	—	—	—
Terpineol	0.8	0.6	1.0	0.5	0.6	0.4	1.1	1.2	0.2	0.5	0.8	0.9	6.1	0.5	0.7
J	0.5	—	0.4	0.2	—	0.2	—	—	—	—	0.4	0.3	—	—	—
K	0.6	0.5	0.3	0.2	0.5	0.3	0.7	0.4	0.1	0.1	0.5	0.4	1.4	0.3	0.5
Terpinylacetat	0.4	0.3	0.4	0.2	0.4	0.2	0.5	0.3	0.1	0.3	0.8	0.4	0.9	0.5	0.5
L	0.4	0.2	0.5	0.1	0.3	0.2	—	—	0.1	1.2	1.8	0.5	1.1	1.3	—
M	0.9	0.5	1.9	0.6	0.6	0.4	1.1	1.6	0.4	0.2	0.8	0.7	0.3	0.6	2.8
N	—	—	—	—	—	0.1	—	0.7	—	—	—	0.1	—	—	1.2

von Bornylacetat und Campher vom Lavandinöl unterscheidet[8]. Bezüglich des Camphergehalts konnten wir diese Regel nicht gänzlich bestätigen, da der Camphergehalt in den intersuchten Ölen sehr verschiedentlich wechselte. Eine bessere Bestätigung konnten wir hinsichtlich der Fraktion, die Borneol, Bornylacetat und Isobornylacetat enthält, erbringen. Bei Lavandinölen wechselt die Summe dieser Komponenten im Bereich von 7.0 bis 11.2% und steigt beim Muster Nr. 8 sogar auf 19.9% an.

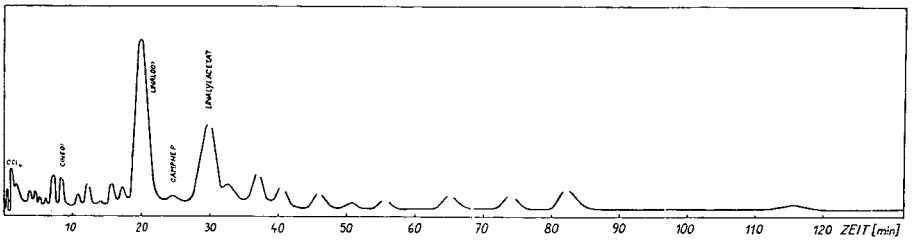


Fig. 3. Chromatogramm des Lavendelöls an Castorwax.

In den ätherischen Ölen des echten Lavendels ist die Konzentration dieser Inhaltsstoffe bedeutend geringer (1.2–1.7%). Die durchgeführten Analysen zeigen weiterhin, dass sich der Gehalt einzelner Komponenten nicht sprungweise ändert, sondern dass es in der Zusammensetzung der Lavandin- und der Lavendelöle oft einen verwischten Übergang gibt, der die festgestellten Regelmässigkeiten zum Verschwinden bringen kann. Wird der Inhalt dieser Stoffe jedoch nicht für einzelne Substanzen, sondern komplex betrachtet, so behält die festgestellte Regelmässigkeit nach wie vor ihre Gültigkeit.

DANK

Für die freundliche Überlassung der Muster von Reinsubstanzen, die uns die Identifikation verschiedener Komponenten aus ätherischen Ölen ermöglichten, sind wir der Leitung der Firma Dragoco und Riedel-De-Haën zu aufrichtigem Dank verpflichtet. Ebenfalls wollen wir für die Überlassung der Muster von untersuchten ätherischen Ölen der Firma Etol (Celje/Jugoslawien) unseren Dank aussprechen.

ZUSAMMENFASSUNG

Die Zusammensetzung verschiedener Muster jugoslawischer als auch einiger französischer Lavendel- bzw. Lavandinöle wurde auf gaschromatographischem Wege bestimmt, wobei in erster Linie die Auftrennungsmöglichkeit für Linalool, Linalylacetat und Campher auf verschiedenen Phasen studiert wurde. Hierbei erwies sich Castorwax als eine ausgezeichnete stationäre Phase, die nicht nur das Linalool-Linalylacetat-Campher-Gemisch, sondern auch die meisten übrigen Komponenten der Lavendelöle einwandfrei aufzutrennen vermag und somit eine gute Beurteilung der Qualität dieser Öle ermöglicht.

SUMMARY

The composition of several samples of Jugoslavian as well as French lavender and lavandin oils was determined by gas chromatography. In the first place the separation

of linalool, linalyl acetate and camphor on various phases was studied. Castor wax proved to be an excellent stationary phase, by means of which not only the mixture linalool–linalyl acetate–camphor can be well separated, but also most of the other components of lavender oil. A good assessment of the quality of these oils is thus possible.

LITERATUR

[1] Y. R. NAVES UND P. TULLEN, *Helv. Chim. Acta*, 43 (1960) 1619.
[2] Y. R. NAVES UND P. TULLEN, *Bull. Soc. Chim. France*, (1960) 2123.
[3] Y. R. NAVES UND P. TULLEN, *Helv. Chim. Acta*, 43 (1960) 2150.
[4] Y. R. NAVES, P. OCHSNER UND P. TULLEN, *Helv. Chim. Acta*, 43 (1960) 1616.
[5] P. A. STADLER, *Helv. Chim. Acta*, 43 (1960) 1601.
[6] P. A. STADLER, A. ESCHENMOSER, E. SUNDT, M. WINTER UND M. STOLL, *Experientia*, 16 (1960) 283.
[7] Y. R. NAVES, *Compt. Rend.*, 246 (1958) 2163.
[8] Y. R. NAVES, *Helv. Chim. Acta*, 42 (1959) 2744.
[9] J. J. BRODERICK, *Aerograph Research Notes*, Fall Issue, 1960.
[10] ANONYM, *Soap, Perfumery, Cosmetics*, 34 (1961) 674, 686.
[11] W. TAGAKI UND T. MITSUI, *Bull. Agr. Chem. Soc. Japan*, 24 (1960) 217.
[12] G. P. CARTONI UND A. LIBERTI, *J. Chromatog.*, 3 (1960) 121.
[13] ANONYM, *Aerograph Research Notes*, Spring Issue, 1961.

STRUKTURANALYSE SILICIUMORGANISCHER VERBINDUNGEN MIT HILFE DER GASCHROMATOGRAPHIE

JAROSLAV FRANC UND JOSEF DVOŘÁČEK

Forschungsinstitut für Organische Synthesen, Pardubice-Rybitví (Tschechoslowakei)

(Eingegangen den 2. September 1963)

Wir waren vor die Aufgabe gestellt in den Destillationsrückständen aus der Herstellung von Methylchlorsilanen die Natur und chemische Struktur der siliciumorganischen Verbindungen zu bestimmen, die die Bestandteile der Destillationsrückstände bilden. Hierbei gerieten wir des öfteren in Verlegenheit, auf welche Weise einige an Si gebundene funktionelle Gruppen zu identifizieren, insbesondere in Fällen, wo es darum ging verschiedene funktionelle Gruppen im gleichen Molekül zu bestimmen. Die sonst so verlässliche Methode der Infrarotspektroskopie versagte hier, da in der Mehrzahl Verbindungen zu bestimmen waren, für die keine verlässlichen Vergleichssubstanzen zur Hand waren. In einzelnen Infrarotspektren zeigten sich wohl charakteristische Banden, aber ihr Aussagewert beschränkte sich bloss auf die Konstatierung der Wahrscheinlichkeit des Vorhandenseins der betreffenden Atomgruppierung. Dazu kam noch, dass einige Bindungsarten keine charakteristischen Frequenzen besitzen.

Schon vor einiger Zeit wurden Versuche zur Identifizierung von an Si gebundenen funktionellen Gruppen durch Abspaltung dieser Gruppen und Bestimmung der Spaltprodukte unternommen. So spalteten BURKHARD UND NORTON[1] Phenyl- oder Alkylgruppen mit konzentrierter Schwefelsäure ab; die hierbei entstandenen aromatischen oder aliphatischen Kohlenwasserstoffe wurden von ihnen massenspektrometrisch identifiziert. KREŠKOV UND BORK[2] und SMITH[3] stellen Übersichten über rein chemische Methoden der Identifizierung von an Si gebundenen Atomgruppen zusammen. Die Gaschromatographie wurde für solche Bestimmungen bisher nur von FRITZ, GROBE UND KSINSIK[4] eingesetzt, der die Gruppe $\equiv$Si–CH_2–Si$\equiv$ durch Chlorierung zu $\equiv$Si–CCl_2–Si$\equiv$ umwandelt und daraus durch Hydrolyse die Methylengruppe in Form von Methylenchlorid (CH_2Cl_2) herausschält, das dann chromatographisch bestimmt wird.

Die Mehrzahl der erwähnten Methoden ist zeitraubend und nicht immer zuverlässig. Wir wandten uns aus diesem Grunde der Ausarbeitung einer raschen und zuverlässigen Methode der chemischen Spaltung von Si–C- oder Si–O–C-Bindungen in Verbindung mit der Gaschromatographie zu.

EXPERIMENTELLER TEIL

Alle Spaltungsreaktionen gehen in einem Mikroreaktor vor sich, der direkt an den Chromatographen angeschlossen ist. Der Mikroreaktor ist in Fig. 1 zu sehen und

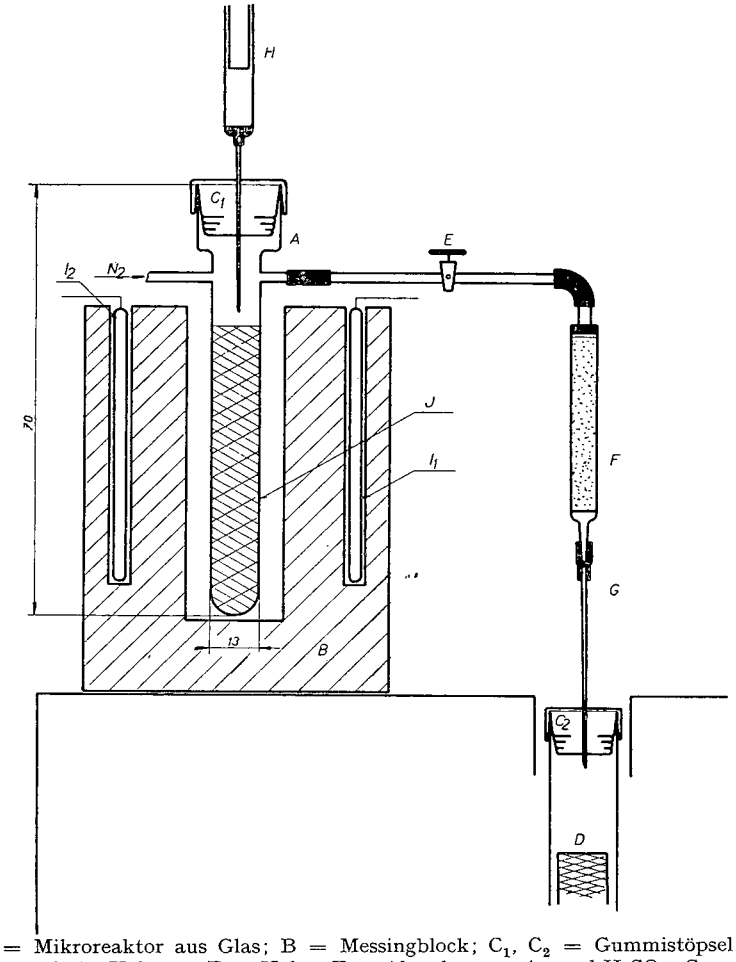


Fig. 1. A = Mikroreaktor aus Glas; B = Messingblock; C_1, C_2 = Gummistöpsel; D = Gaschromatographische Kolonne; E = Hahn; F = Absorber von Aerosol H_2SO_4; G = Spritznadel; H = Spritze; I_1, I_2 = Heizkörper; J = Reaktionsgemisch.

bedarf keiner weiteren Erläuterung. Durch einmaliges Öffnen des Hahnes zwischen Mikroreaktor und Chromatographen während 10–15 Sekunden werden die flüchtigen Spaltprodukte in die Kolonne eingegeben. Als Chromatograph wurde das Gerät von Griffin & George mit Wärmeleitungsdetektor verwendet.

Alkylsilane und Alkylsiloxane

(a) Spaltung mit konzentrierter Schwefelsäure (Dichte 1.834), die mit V_2O_5 gesättigt ist. Temperatur im Mikroreaktor: 175°. Dauer der Spaltung: 15–20 Min.

(b) Chromatographische Bestimmung der Spaltprodukte: Kolonne 180 cm gefüllt mit Silikagel (Uebeticon), 30–40°, Trägergas Stickstoff 2.9 l/Std.

Arylsilane und Arylsiloxane

(a) Spaltung wie bei Alkylsilane.

(b) Chromatographische Bestimmung der Spaltprodukte: Kolonne 180 cm ge-

füllt mit Rysorb + 20 % Destillationsrückstand aus der Destillation von Methylphenylsilikonöl, 80°, Trägergas Stickstoff 2.9 l/Std.

Chloralkylsilane und Chloralkylsiloxane

(a) Spaltung wie bei Alkylsilane.

(b) Chromatographische Bestimmung der Spaltprodukte: Kolonne 180 cm gefüllt mit Rysorb + 20 % Destillationsrückstand aus der Destillation von Methylphenylsilikonöl, 20°, Trägergas Stickstoff 2.9 l/Std.

Alkoxysilane

(a) Spaltung mit Jodwasserstoffsäure (Dichte 1.70) bei 75°.

(b) Chromatographische Bestimmung der Spaltprodukte: Kolonne 180 cm gefüllt mit Rysorb + 20 % Destillationsrückstand von Methylphenylsilikonöl, 40°, Trägergas Stickstoff 2.9 l/Std.

Vinylsilane

(a) Zunächst wird Chlor addiert durch Sättigen von Chlor im Vinylsilan während 60 Min. auf dem Wasserbad. Danach wird wie im Fall der Alkylsilane vorgegangen, d.h. Spalten mit konz. Schwefelsäure bei 175°.

(b) Chromatographische Bestimmung der Spaltprodukte: Kolonne 180 cm gefüllt mit Rysorb + 20 % Destillationsrückstand von Methylphenylsilikonöl, 20°, Trägergas Stickstof 2.9 l/Std.

≡Si–Si≡ und ≡Si–H-Bindungen

(a) Spaltung mit 25 %-iger KOH bei H-Silanen schon in der Kälte, bei Disilanen bei 100°.

(b) Chromatographische Bestimmung der Spaltprodukte: Kolonne 90 cm gefüllt mit Aktivkohle (Supersorbon), 20°, Trägergas Stickstoff 2.9 l/Std.

In allen angeführten Fällen betrug der Durchmesser der chromatographischen Kolonne 5 mm und die Korngrösse der Träger der stationären Phase 0.3–0.4 mm.

DISKUSSION DER ERGEBNISSE

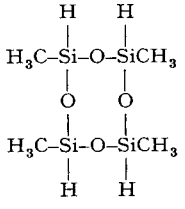
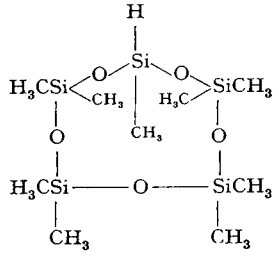
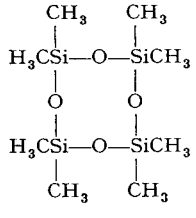
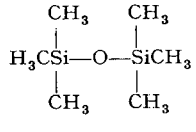
Die grösste Schwierigkeit, der wir beim Aufschluss der siliciumorganischen Substanzen gegenüberstanden, war die schlechte Mischbarkeit der Proben mit den hydrophilen Reagentien, die zum Aufschliessen benutzt wurden. Das zeigte sich besonders bei den Monomeren, die bei höherer Temperatur leicht flüchtig wurden; nur die Polymeren ergaben ohne irgendwelche Vorsichtsmassnahmen richtige Resultate. Um auch bei Monomeren richtige Ergebnisse zu bekommen tränkten wir poröses Material (in unserem Falle wurde als solches Rysorb verwendet, das für gewöhnlich als Trägermaterial für die stationäre Phase in der Gaschromatographie dient) mit der zu untersuchenden Substanz und überschichteten sodann mit dem aufschliessenden Reagens. Infolge der grossen Berührungsfläche geht die Reaktion rasch unter Entwicklung einer genügenden Menge an Spaltprodukten vor sich.

Die Zerstörung der Si–C-Bindung wird — wie aus dem experimentellen Teil zu ersehen ist — in der Mehrzahl der Fälle mit Schwefelsäure bewerkstelligt, mit der das Spalten meist gut verläuft. Bei Silikonen mit mehr Chloratomen hat sich eine Zugabe

TABELLE I

ALKYLSILANE UND ALKYLSILOXANE

+ + + grosse Menge; + + mittlere Menge; + kleine Menge; + — Spuren; — — abwesend

Relative Retentionszeiten	H_2 *0.11*	CH_4 *0.20*	C_2H_6 *0.56*	CO_2 *1.00*	C_3H_8 *1.95*
$CH_3Cl_2SiCH_2SiCl_2CH_3$	+ —	+ + +	— —	+ + +	— —
$CH_3Cl_2SiSiCl_2CH_3$	+ + +	+ + +	— —	+ + +	— —
$(CH_3)(C_2H_5)SiCl_2$	+ —	+ + +	+ + +	+ + +	— —
$(C_3H_7)_2SiCl_2$	— —	— —	— —	+ + +	+ + +
$(C_3H_7)_2SiCl_2 + (C_3H_7)SiCl_3$	— —	— —	— —	+ + +	+ + +
	+ + +	+ + +	— —	— —	— —
	+ + +	+ + +	— —	— —	— —
$(CH_3)_2SiCl_2$	— —	+ + +	— —	+ +	— —
$(C_2H_5)_2SiCl_2$	— —	— —	+ + +	+ + +	— —
$(CH_3)(C_3H_7)SiCl_2$	— —	+ + +	— —	+ + +	+ + +
CH_3SiCl_3	+	+ + +	— —	+ + +	— —
$(CH_3)_2ClSiCH_2SiCl_2CH_3$	+ —	+ + +	— —	+ + +	— —
$CH_3Cl_2SiCH_2CH_2SiCl_2CH_3$	+ —	+ + +		+ + +	
$C_2H_5SiCl_3$	+ —	+ + +	+ +	+ + +	
	— —	+ + +		+	
	— —	+ + +		+ —	
$(CH_3)_3SiSi(CH_3)_3$	+ +	+ + +		+ + +	
$[(CH_3)_2(C_2H_5)Si]_2O$	+ —	+ + +	+ + +	+	
$(CH_3)_2ClSiSiCl_2C_2H_5$	+ +	+ + +	+	+ + +	

von etwas V_2O_5 zur Schwefelsäure bewährt; in solchen Fällen empfiehlt es sich die Spaltung direkt auf dem porösen Material unter Zugabe eines Tropfens Wasser auszuführen. Die Zerstörung der Si–C-Bindung verläuft so leichter.

Das Reaktionsprodukt aus der Spaltung von Alkylsilanen und Alkylsiloxanen sind die zugehörigen Kohlenwasserstoffe, deren gaschromatographische Identifizierung eine leichte Sache ist. Der hier beschriebene Gang der Identifizierung von einzelnen siliciumorganischen Verbindungen aus Gemischen ist besonders in solchen Fällen vorteilhaft anwendbar, bei denen an Si verschiedene Alkyle gebunden sind.

Die Leichtigkeit, mit der die Zerstörung der Bindung Si-C verläuft ist verschieden; Arylsilane und Arylsiloxane spalten leichter Benzol ab, währenddem der zugehörige Paraffinkohlenwasserstoff bei Alkylsilanen schwerer abspaltbar ist. Aus Gründen der methodischen Einheitlichkeit haben wir dort, wo angängig, die Spaltung mit konzentrierter Schwefelsäure beibehalten, wenn auch CHVALOVSKÝ UND BAŽANT[5] beweisen, dass die Spaltung von Arylsilanen mit 60–90 % am besten verläuft. In allen Fällen entsteht für unsere Zwecke immer noch genug an aromatischem Kohlenwasserstoff.

In der Seitenkette chlorsubstituierte Chloralkylsilane und Chloralkylsiloxane spalten mit Schwefelsäure die entsprechenden Chlorparaffine ab.

In der Tabelle I sind noch weitere Eigenheiten, die bei der Abspaltung von Alkylen auftreten zu sehen. Bei Disilanen Si–Si und bei Hydrosilanen Si–H entsteht neben Alkyl ausserdem Wasserstoff in ansehnlicher Menge, desgleichen entsteht bei Silikonen mit Chlor oder Äthoxyl Kohlendioxyd. Interessant ist auch, dass bei der Spaltung von $(C_2H_5)SiCl_3$ neben Äthan auch Methan auftritt, wenn vorher nicht zur Hydrolyse ein Tropfen Wasser zugegeben wurde.

Am Kern chlorsubstituierte Arylsilane (Tabelle II) spalten die chlorsubstituierten Aromaten als Ganzes ab (z.B. Chlorbenzol), aber die Reaktion geht nicht mehr so leicht wie bei nicht substituierten Arylsilanen vonstatten. Bei Chlorbenzylderivaten konnten wir unter den von uns gewählten Arbeitsbedingungen überhaupt keines der zugehörigen Chlortoluole erhalten.

Im Gegensatz dazu spalten Chloralkylsilane leicht die Chloralkylgruppe, meist in Form von Methylchlorid — auch bei höheren Chloralkylen — ab, wie aus der Tabelle III zu entnehmen ist. Man kann auch noch die Stellung des Chlors im Alkyl bestimmen (siehe z.B. $CH_3CHClSi(OC_2H_5)_3$ und $CH_2ClCH_2Si(OC_2H_5)_3$); im ersten der beiden hier angeführten Beispiele entsteht hauptsächlich Methylchlorid und weniger Äthylchlorid, währenddem im zweiten Fall es gerade umgekehrt ist. Selbstverständlich lassen sich Alkyl- und Chloralkylgruppen ohne Beschwerlichkeit nebeneinander identifizieren.

Zur Identifizierung von Alkoxygruppen (Tabelle IV) nebeneinander wandten schon früher einige Autoren[6–8] gaschromatographische Methoden an, die sich auch bei der Identifizierung von an Si gebundenen Alkoxygruppen bewährten.

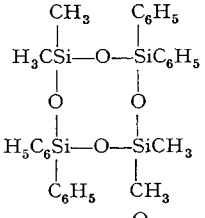
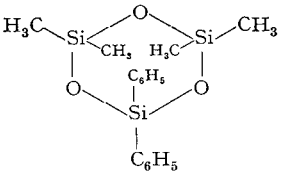
Bei vinylsubstituierten Silanen (Tabelle V) konnten wir begreiflicherweise mit unserer Spaltungsmethode nicht den zugehörigen ungesättigten Kohlenwasserstoff fassen; wir griffen deshalb zu einer Behelfsreaktion, nach der zunächst an die Doppelbindung Chlor angelagert wird. Das entstandene Chlorderivat wird sodann der Spaltung unterworfen, bei der Alkylchlorid entsteht.

Wir versuchten auch die Identifizierung von OH-Gruppen durch Ersatz des Wasserstoffs der OH-Gruppe mit metallischem Natrium, mussten dabei aber feststellen, dass es nicht immer gelang die Apparatur vollkommen trocken zu bekommen,

TABELLE II

ARYLSILANE UND ARYLSILOXANE

+ + + grosse Menge; + + mittlere Menge; + kleine Menge; + — Spuren; — abwesend

	H_2	O_2	CH_4	CO_2	X^*	C_6H_6	C_6H_5Cl
Relative Retentionszeiten	*0.2*	*0.3*	*0.5*	*1.0*	*1.9*	*4.9*	*20.4*
$(C_6H_5)_2SiCl_2$	+ —	+ —		+ + +		+ +	
$(C_6H_5)_2(CH_3)_2Si$			+ + +	+ + +		+ + +	
$[(C_6H_5)_2(CH_3)Si]_2O$	+ —	+ —	+	+ + +	+	+ + +	
$(C_6H_5)SiCl_3$	+	+		—		+ + +	
$(C_6H_5)_3SiOSi(CH_3)_3$			+ + +	—	+	+ + +	
$(C_6H_5)_2Si(OH)_2$	+ +	+	—	—	+	+ + +	
$ClC_6H_4Si(CH_3)_3$			+ + +	—			+ +
$CH_3SiO[Si(C_6H_5)_2O]_2Si(CH_3)_3$			+ + +			+ + +	
			+ + +	—	—	+ + +	
	+ +		+ + +	—		+ + +	

* X = unbekannt.

TABELLE III

CHLORALKYLSILANE UND CHLORALKYLSILOXANE

+ + + grosse Menge; + + mittlere Menge; + kleine Menge; — abwesend

	H_2	CH_4	CO_2	CH_3Cl	C_2H_5Cl
Relative Retentionszeiten	*0.2*	*0.6*	*1.0*	*3.47*	*8.65*
$CH_3CHClSi(OC_2H_5)_3$	+ +	+*	+ + +	+ + +	+
$ClCH_2CH_2Si(OC_2H_5)_3$	+ +	—*	+ + +	+ +	+ + +
$ClCH_2Si(OC_2H_5)_3$			+ + +	+ + +	
$ClCH_2CH_2CH_2SiCl_2(CH_3)_2$		+	+ +	+ + +	
$ClCH_2(CH_3)_2SiOSiCl_2CH_2Cl$		+ + +	+ +	+ +	
$ClCH_2SiCl(CH_3)_2$		+ + +	+ + +	+ + +	
$ClCH_2SiCl_3$			+ + +	+ + +	

* Festgestellt an der Kolonne mit SiO_2.

TABELLE IV

ALKOXYSILANE

+ + + grosse Menge; —— abwesend

	CH_3J	C_2H_5J
Relative Retentionszeiten	*1.00*	*2.43*
$(CH_3)(CH_2{=}CH)Si(OC_2H_5)_2$	——	+ + +
$CH_3Si(OC_2H_5)_3$	——	+ + +
$ClCH_2CH_2Si(OC_2H_5)_3$	——	+ + +
$ClC_6H_4CH_2Si(OC_2H_5)_3$	——	+ + +
$CH_3CHClSi(OC_2H_5)_3$	——	+ + +
$CH_2{=}CHSi(OCH_3)_3$	+ + +	——

TABELLE V

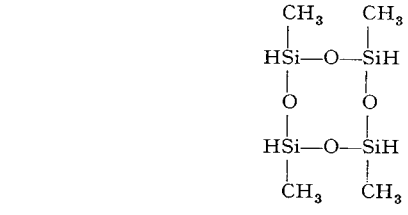
VINYLSILANE

	C_2H_5Cl
$CH_2{=}CHSi(OC_2H_5)_3$	grosse Menge
$CH_2{=}CHSiCl_3$	grosse Menge

TABELLE VI

VERBINDUNGEN MIT ≡Si–Si≡ UND ≡Si–H BINDUNGEN

+ + + grosse Menge

	$H_2(20°)$	$H_2(100°)$
CH_3SiHCl_2	+ + +	
CH_3SiH_2Cl	+ + +	
	+ + +	
$(CH_3)Cl_2SiSiCl_2(CH_3)$		+ + +
$(CH_3)_2ClSiSiCl(CH_3)_2$		+ + +
$(CH_3)_2ClSiSiCl_2(C_2H_5)$		+ + +
$(C_2H_5)_2SiHCl$	+ + +	
$(C_2H_5)_2ClSiSiCl_2(C_2H_5)$		+ + +

sodass ein allenfalls festgestellter Gehalt an Wasserstoff kein sicheres Zeichen war für die Anwesenheit der OH-Gruppe in der untersuchten Substanz.

Eine Unterscheidung zwischen Si–H- und Si–Si-Bindungen (Tabelle VI) ist durchführbar, wenn man die Reaktionsgeschwindigkeit bei der Spaltung mit Lauge verfolgt. Das kann so geschehen, dass man den Mikroreaktor mehrmals hintereinander mit dem Trägergas durchspült. Ist Si–H zugegen, dann sinkt mit fortschreitendem Durchspülen die Menge an entbundenem Wasserstoff. Bei Si–Si-Bindungen ist das Gegenteil der Fall. Die Spaltung von Si–Si-Bindungen verläuft in manchen Fällen nur bei höherer Temperatur.

Abschliessend lässt sich zusammenfassend sagen, dass man mit Hilfe der Gaschromatographie bequem und in kurzer Zeit alle für gewöhnlich in Betracht kommenden funktionellen Atomgruppierungen bei Silikonen identifizieren kann, gegebenenfalls auch die Bindungsverhältnisse zwischen den Silicium- und Kohlenstoffatomen.

ZUSAMMENFASSUNG

Es wurde eine Methode zur Identifizierung funktioneller Gruppen und einiger Bindungsarten von siliciumorganischen Verbindungen ausgearbeitet; die Methode besteht darin, dass in einem an den Gaschromatographen angeschlossenen Mikroreaktor die zu analysierende Verbindung reagiert und die flüchtigen Reaktionsprodukte unmittelbar in den Chromatographen übergeführt werden. Auf diese Weise lassen sich Alkyl-, Chloralkyl-, Phenyl-, Chlorphenyl-, Alkoxy- und Vinylgruppen sowie ≡Si–Si≡ und ≡Si–H in siliciumorganischen Verbindungen rasch nachweisen.

SUMMARY

A method has been developed for the identification of functional groups and some types of bonding in organic silicon compounds. The compound being analysed is subjected to a degradation reaction in a micro-reactor, which is connected to a gas chromatograph, so that the volatile reaction products pass directly into the gas chromatograph. In this way alkyl, chloroalkyl, phenyl, chlorophenyl, alkoxy and vinyl groups, as well as ≡Si–Si≡ and ≡Si–H, can be rapidly detected in organic silicon compounds.

LITERATUR

[1] CH. A. BURKHARD UND F. S. NORTON, *Anal. Chem.*, 21 (1949) 304.
[2] A. P. KREŠKOV UND V. A. BORK, *Usp. Khim.*, 28 (1959) 576.
[3] J. C. B. SMITH, *Analyst*, 85 (1960) 465.
[4] G. FRITZ, J. GROBE UND D. KSINSIK, *Z. Anorg. Allgem. Chem.*, 302 (1959) 175.
[5] V. CHVALOVSKÝ UND V. BAŽANT, *Collection Czech. Chem. Commun.*, 26 (1961) 282.
[6] M. VERTALIER, *Chim. Anal. (Paris)*, 40 (1958) 80.
[7] T. R. CROMPTON, *Analyst*, 86 (1961) 652.
[8] K. KRATZL UND K. GRUBNER, *Monatsh.*, 89 (1958) 618.

MICRODETERMINATION OF DITHIOCARBAMATES BY GAS CHROMATOGRAPHY*

CORRADO BIGHI
Istituto Chimico dell' Università, Ferrara (Italy)

(Received September 9th, 1963)

During previous work at this institute[1], carbon disulphide liberated by the decomposition of dithiocarbamic acid derivatives with hot acid was determined by gas chromatography, using the method of internal normalisation[2].

This method involved:

(a) the use of a second component, in this case *n*-heptane, which however gave a rather long retention time (28 min) under the experimental conditions employed;

(b) the weighing of volatile products;

(c) the injection of the sample into the gas chromatography apparatus by means of a microsyringe. This is not always easy or reproducible, especially when the materials undergoing quantitative microanalysis possess high vapour pressures;

(d) the use of an experimentally determined correction factor in calculating the concentration of the product being examined.

It is therefore particularly desirable to replace the absorption of CS_2 in *n*-heptane by condensation of the free CS_2 in a trap immersed in liquid air.

Use of this direct method makes gas chromatography of the CS_2 evolved more accurate and more sensitive, and at the same time overcomes the difficulties listed above.

Elimination of the second component, and hence of any impurities it might contain, is particularly desirable, especially if these impurities include the substance to be estimated[3].

In the procedure adopted in the present work, CS_2 obtained from the decomposition of dithiocarbamic acid derivatives by hot H_2SO_4 was collected in a liquid air trap, omitting the intermediate operations. The whole of this condensate was then introduced directly into the gas chromatography apparatus. This method permits the determination of very small quantities of CS_2 (considerably smaller than those which can be with equal ease determined by other methods), according to the sensitivity of the detector and to the accuracy in measuring the areas under the recorded peaks[4].

The method described below permits the determination, in solutions of various dithiocarbamates, of amounts of CS_2 as low as a few γ, under conditions which eliminate sources of error, such as weighing and sampling by means of a microsyringe.

* Paper presented at Bologna to the Emilian Group of the Società Chimica Italiana on July 14th, 1962.

EXPERIMENTAL

Calibration curves

The first step was to construct calibration curves for pure CS_2.

Owing to its extreme volatility, pure CS_2 could not be injected directly into the column, since the peak would run off the scale, even if the smallest samples were used and the apparatus was set for the lowest sensitivity.

It was therefore necessary to prepare a number of solutions of CS_2 in *n*-heptane, not by weighing, but by volumetric measurement with a microsyringe. Using 0.005–0.01 ml of these solutions, it was then possible to inject a series of known amounts of CS_2 into the column and to measure the areas under the corresponding peaks.

The results used to prepare the calibration curve, corresponding to a flow rate of the carrier gas equal to 1.5 l/h, are shown in Table I.

TABLE I

DATA USED TO PREPARE THE CALIBRATION CURVE WITH A FLOW RATE OF 1.5 l/h

CS_2 concentration ($g \times 10^{-5}$)	*Area under peak (cm^2)*
1.261	3.5
2.522	6.6
3.783	10.4
6.305	17.8
11.349	31.6
12.610	35.0
18.915	52.0
25.220	69.6
37.830	104.0
50.440	140.0

The areas, which were measured planimetrically, were obtained under the conditions shown in Table II.

The calibration curve shown in Fig. 1 is suitable for the determination of medium and high concentrations. It cannot, however, be used for low concentrations owing to the irregularity of the peaks obtained, which are dragged out too much by the slowly flowing carrier gas. To obtain regular peaks with areas coinciding exactly

TABLE II

EXPERIMENTAL CONDITIONS

Equipment	Fractovap model B (C. ERBA-MILAN)
Column	2 m length, i.d. 6 mm
Packing	30–60 mesh celite
Liquid phase	25% silicone grease E-301
Thermostat chamber temperature	85° ± 0.2
Carrier gas	He, at 1.5 l/h 3.6 l/h
Bridge current	18 mA
Chart speed	1.25 cm/min
Retention times	9 min 45 sec at 1.5 l/h 4 min at 3.6 l/h

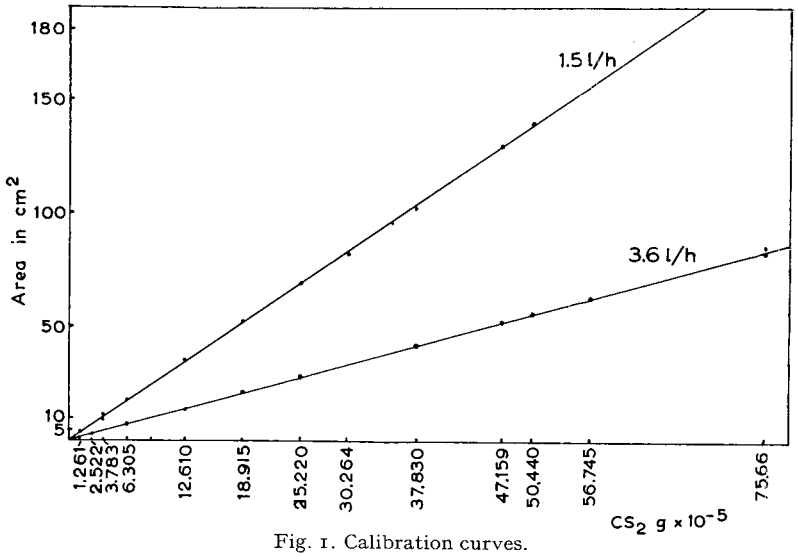


Fig. 1. Calibration curves.

with the calibration curve, the flow rate of the carrier gas was therefore increased from 1.5 to 3.6 l/h for low concentrations of CS_2.

The new curve, which is also shown in Fig. 1, possesses a less steep slope than the first plot and has the advantage that it can be used for all the concentrations examined. The data used to construct it are shown in Table III.

The experimental conditions mentioned above remained unchanged, except that the rate of flow of the carrier gas was increased from 1.5 to 3.6 l/h, the pressure at the column inlet being equal to 0.27 kg/cm². This change reduced the retention time of carbon disulphide to 4 min.

TABLE III

DATA USED TO PREPARE THE CALIBRATION CURVE WITH A FLOW RATE OF 3.6 l/h

CS_2 concentration ($g \times 10^{-5}$)	*Area under peak (cm^2)*
0.448	0.52
2.522	2.8
6.305	7.2
12.610	14.0
18.915	20.8
25.220	28.0
37.830	42.0
50.440	55.6
56.745	63.2
75.660	84.0

Determination of CS_2

The above calibration curves were used to determine, by gas chromatography, the CS_2, produced from the decomposition of specially prepared pure sodium ethylene-bis-dithiocarbamate with hot H_2SO_4.

This compound possesses the great advantage over the dithiocarbamates of other metals in being water soluble. Standard aqueous solutions could thus readily be prepared, with concentrations ranging from 4 to 12 %. Solutions containing various amounts of CS_2 were then prepared by diluting known volumes of these solutions to the required concentrations.

The apparatus used to distil the samples is shown in Fig. 2 on a scale of 1:10. The operation is carried out in a stream of helium (aspirated in directly from a fractometer) in place of air, to avoid any possible oxidation of the products during decomposition and distillation.

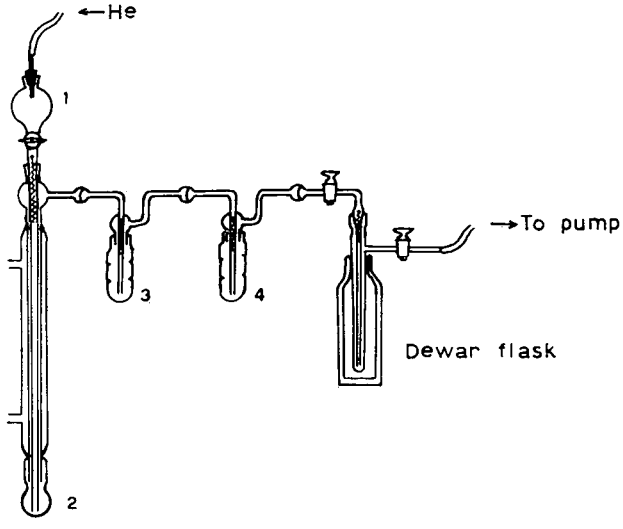


Fig. 2. Apparatus for distillation of CS_2.

The rate of flow of He must not be too high, and should be 1 l/h. Traps 3 and 4 contain concentrated H_2SO_4 to retain as much as possible of the water vapour evolved in the distillation. The cadmium acetate tube included in Clarke's apparatus to absorb hydrogen sulphide has been omitted, since H_2S has a much shorter retention time and does not interfere at all with the detection of CS_2.

The cell in which the CS_2 is trapped and solidified by liquid air is illustrated in Fig. 3.

The evaporated sample is introduced into the gas chromatography apparatus by displacing a known quantity from a calibrated container, using the same carrier gas. To ensure that the resultant peaks are regular and significant, and therefore easily measurable, the gaseous sample should be introduced into the adsorption column as rapidly as possible, and without excessive dilution by the carrier gas. The gas will thus occupy the entire cross-section of the column, but will spread over the shortest possible portion of its length. For a column with an internal diameter of 4–8 mm, the volume of the gaseous sample should normally not exceed 5 cc. In the present work it was found that an excessive rate of flow of the carrier gas could be avoided under the existing experimental conditions, and the CS_2 could be displaced

sufficiently rapidly (assuming evaporation from the cell at ambient temperature), if the total internal volume of the cell was 3, or at most 4 cc.

On the other hand, the condensation cell must be at least 15–20 cm long to ensure complete collection of the CS_2 during distillation.

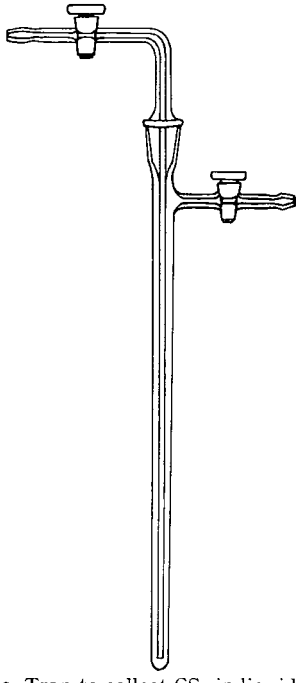


Fig. 3. Trap to collect CS_2 in liquid air.

It is not necessary to known the exact volume of the cell nor the pressure and temperature of the gas retained in it, since all the gaseous products liberated by decomposition and distillation of a known quantity of the initial substance are eventually displaced by the carrier gas and carried into the adsorption column.

The method of carrying out the entire operation in the various stages (decomposition, distillation and introduction of the condensate into the column) is as follows. The required volume of the solution to be examined is introduced into flask 2, and 10–20 ml of 1.1 *N* sulphuric acid, previously heated to its boiling point, are placed in funnel 1. The funnel is then closed with a stopper through which passes the inlet tube for helium from the fractometer. Slight suction is then started using the pump, and the flow is adjusted by means of a Mohr clip to the desired velocity as soon as all the acid has passed into the flask. The solution is immediately heated to 100°, so that the liquid begins to boil evenly as soon as possible.

After 50 min distillation, the taps of the cell containing the condensed CS_2 and other possible volatile products are closed, the cell is removed from the Dewar flask, and is immediately attached via a suitable adaptor to the manifold for the introduction of the sample into the chromatographic column (Fig. 4).

It is very important that this operation should be completed fairly quickly,

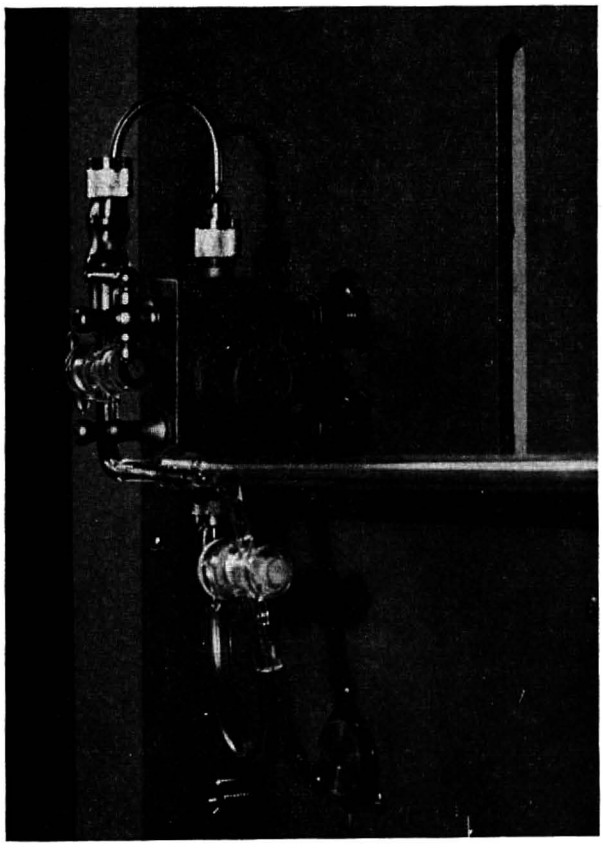


Fig. 4. The manifold for the introduction of samples.

since the stream of helium which is to carry CS_2 into the column must be connected while the cell is still very cold, to avoid evaporation of any water condensed in the cell. Introduction of water vapour into the column would give rise to a peak close to that of the CS_2, so that the latter could not be perfectly zeroed.

If, on the other hand, the temperature of the cell remains too low, the CS_2 may not be completely vaporised. It is therefore advisable to allow the cell to warm up for 2 min after its removal from the Dewar flask before the carrier gas is passed in. About 1 min after connecting the carrier gas, the CS_2 cell is again cut off from the analytical system, to avoid contamination of the column with evaporating water.

Disregarding the limits of sensitivity of the apparatus and of the planimetric measurements, it can be stated with considerable confidence that the method does not give appreciable errors.

In addition to the work with pure water-soluble sodium dithiocarbamate, the method was also tried out on the corresponding zinc, manganese, and iron salts. Since these are insoluble in water, very dilute aqueous suspensions of these compounds were prepared, adding a few drops of a wetting agent (polyglycol esters) to facilitate the dispersion process.

In contrast to the use of the same dithiocarbamates suspended in inert diluents, or to the use of the solid product alone, the addition of a wetting agent enabled the control tests to be carried out on quantities of the order of a few γ, by reliable volumetric sampling, and with perfectly reproducible results.

It can be seen from Table III that with the aid of the procedure described it is possible to determine quantities of dithiocarbamates corresponding to less than 5 γ of CS_2, even if the parent compounds are rich in impurities and do not dissolve in water. Control experiments with pure products gave, even at lower concentrations, perfectly reproducible results which were practically identical with theoretical values.

Ethylene-bis-dithiocarbamic derivatives tend to give side-reactions which effectively reduce the quantity of CS_2 evolved by the production of other sulphur compounds, such as ethylenethiourea and H_2S. To avoid this source of error it is imperative to carry out the distillation at 100° throughout.

Having eliminated interference directly associated with the final processes of evaluation of CS_2, the conversion to CS_2 of even extremely small amounts of organic dithionic compounds, irrespective of their composition and nature (provided they contain the $-\overset{\diagup\!\!\diagdown S}{C}-S-$ group which can be converted to $C\ll^{S}_{S}$), can be controlled by controlling the conditions under which the starting materials are decomposed.

SUMMARY

Carbon disulphide obtained from the acid decomposition of pure and commercial dithiocarbamates and bis-dithiocarbamates was entrained in a stream of helium and condensed in a trap immersed in liquid air.

The entire condensate was then analysed by removing the trap from the liquid air and connecting it directly to an appliance for introducing gaseous substances into a gas chromatographic column.

The experimental procedures for preparing the calibration curves and for the determination of the compounds in question are reported, and advantages of the proposed method are described. One of these advantages is that, besides eliminating any risk of interference which may be encountered in other methods, this technique enables amounts down to a few γ of carbon disulphide to be determined.

With the aid of the method described it is therefore possible to determine quantitatively trace amounts of dithiocarbamates, or of any compounds which can liberate carbon disulphide.

REFERENCES

[1] C. BIGHI, *Atti Accad. Sci. Ferrara*, 38 (1961) 1.
[2] D. G. CLARKE, H. BAUM, E. STALEY AND W. F. HESTER, *Anal. Chem.*, 23 (1951) 1842.
[3] L. R. KILEY AND R. T. SCHEDDEL, *Anal. Chem.*, 31 (1959) 157.
[4] F. J. VILES, *J. Ind. Hyg. Toxicol.*, 22 (1940) 188.

PHOTOIONIZATION DETECTOR FOR GAS CHROMATOGRAPHY

I. DETECTION OF INORGANIC GASES

MIKIYA YAMANE

Hitachi Central Research Laboratory Kokubunji, Tokyo (Japan)

(Received October 8th, 1963)

Two previous papers[1,2] described properties of the argon ionization detector, in which the radioactive source is replaced by a gaseous discharge in the vicinity of the sensing electrodes. It was shown that the detector was based on the same reactions as those of LOVELOCK's argon ionization detector[3], except that primary electrons were supplied from the discharge.

There has been, however, added interest in this type of device since the discharge excited in inert gases emits ultraviolet radiation with energies above the ionization potentials of solute molecules. This implies that the radiation can produce ionization when passing through the solute gases. This fact has been made the basis of a new detector by LOVELOCK[4], but little information on the performance characteristics of the detector has been published.

The present work has been directed toward finding a practical photoionization detector by modifying its predecessors[2]. The modification was made in such a way that only light emitted from the discharge can pass through the sensing region while charged particles generated by the discharge are inhibited from flowing to the sensing electrodes.

The detector as modified was operated using helium as both the discharge and the carrier gas, and it was found that the photoionization method can be applied with considerable advantages over other techniques to the detection of wide variety of substances and that it is particularly well suited to the investigation of inorganic gases, the detection of which is difficult by other techniques when only small quantities are present.

APPARATUS

Figs. 1(A) and 1(B) show the photoionization detector used, a modified version of those previously described[2]. The detector consists of two glass chambers, one of which is the discharge chamber and the other the sensing chamber, and is provided with the inlets for the discharge and the carrier gas and a common outlet. In the discharge chamber is mounted a pair of 0.5 mm Kovar wires, the ends of which are bent toward each other (gap distance approximately 0.1 mm), so that the discharge may be fixed there. The anode in the sensing chamber is made of a Kovar pipe of 1.5 mm o.d. and 1 mm i.d. The cathode is a semi-spherical nickel dish with a radius of 2.5 mm.

The two chambers are joined together by a funnel-shaped Kovar pipe, so that the

light beam from the discharge may traverse through the space between the sensing electrodes. The light, however, does not irradiate the surface of the sensing electrodes and thus photoelectric emission from the sensing electrodes is negligible. This pipe is electrically grounded and acts as an electrode that prevents charged particles from flowing into the sensing chamber (hereafter this pipe is called the ion trap).

a

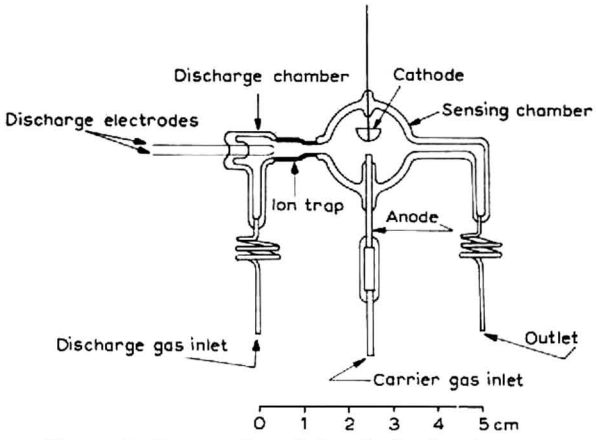


Fig. 1 (A). Construction of photoionization detector.

b

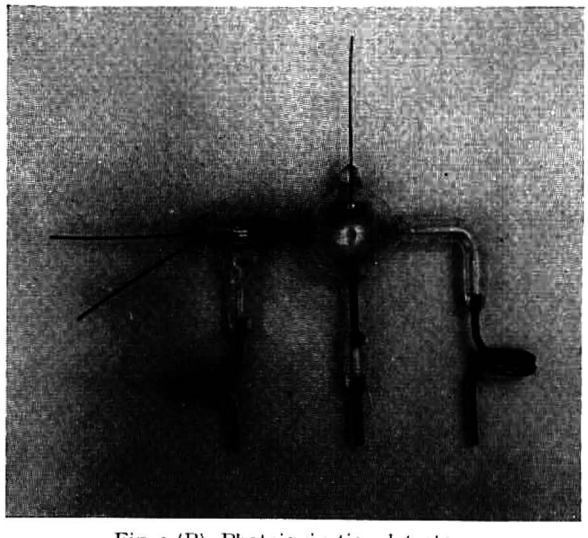


Fig. 1 (B). Photoionization detector.

The basic arrangement of the apparatus used for measuring the photoionization current of solute molecules is the same as that described in the previous paper[2]. The apparatus incorporates two separate gas flows, namely, the discharge gas flow and the carrier gas flow. In all the measurements helium was used as both the discharge and the carrier gas for two reasons.

(1) As discovered by HOPFIELD[5] and studied by TANAKA *et al.*[6-8], the discharge in helium at high pressures emits a continuum of radiation in the wavelength region

between *ca.* 600 and 1100 Å, photons of the highest energies that can be obtained under the simple discharge condition. This continuum can profitably be used to ionize all kinds of molecules except those of helium and neon.

(2) These photons should not be absorbed by the carrier gas, as this would reduce the ionization efficiency of the solute gases. The use of helium as the carrier gas satisfies this requirement and, moreover, should not give any background current in the absence of solute gases.

Precautions were taken to remove impurities from helium by incorporating an impurity trap into each of the gas flows. These traps were copper columns (1 m × 4 mm i.d.) packed with activated charcoal. After activation (by heating to 200° for about 1 h), they were immersed in liquid nitrogen.

EXPERIMENTAL RESULTS

Background current

In principle, the background current of the photoionization detector should be zero in the absence of solute gases, since charged particles generated by the discharge are inhibited from flowing into the sensing chamber. In order to check this prediction, measurements were made of the background current–anode voltage characteristic under the following conditions:

Carrier gas	= He 30 ml/min.
Discharge gas	= He 0, 50, 100, 150 ml/min.
Discharge current	= 30 μA.
Ion trap voltage	= 0 V.
Impurity trap	= Activated charcoal 1 m (—196°).
Column	= Molecular sieve 13X 1.5 m (20°).

The results plotted in Fig. 2 show that the background current in the saturation region is of the order of 10^{-10} to 10^{-9} A.

It can be seen that all the curves pass through the origin, indicating that the effect of the electrical force between the discharge and the sensing chamber[2] is entirely eliminated by the earthed ion trap.

It is clear from Fig. 2 that the background current varies with the discharge gas flow, and that in the four curves a maximum current is obtained at a flow rate of 50 ml/min. To investigate the matter further, gas-flow dependence studies were made under the same conditions, while the anode voltage was kept constant and the gas flow rate was varied. The results of measurements at three different anode voltages are shown in Fig. 3. The curves in Fig. 3 show that with increasing gas flow the background current increases at first, reaches a maximum at *ca.* 50 ml/min and then decreases. This trend of the curves is not in accordance with the previous investigations where the background current increased continually with the gas flow. It can be interpreted in terms of photoionization of impurities which, in spite of purification by the cold impurity traps, are entrained by both the carrier and the discharge gas.

There are two possible causes for the initial rise of the background current. (1) The discharge gas is contaminated with impurities that come from the wall of the tube or from the impurity trap. These impurities when present between the discharge and the sensing chamber can absorb photons, thus reducing the intensity of ultra-

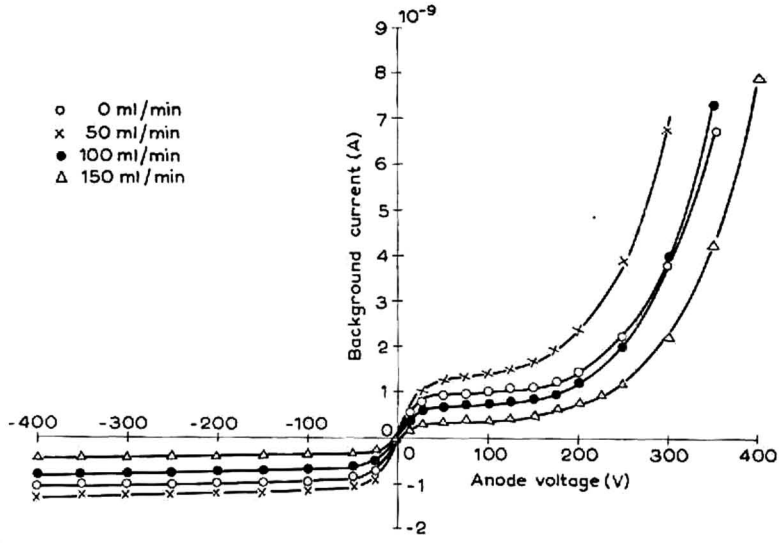


Fig. 2. Background current as a function of the anode voltage. Carrier gas = He 30 ml/min; discharge gas = He 0, 50, 100, 150 ml/min; discharge current = 30 μA; impurity trap = activated charcoal (—196°); column = molecular sieve 13X 1.5 m (20°).

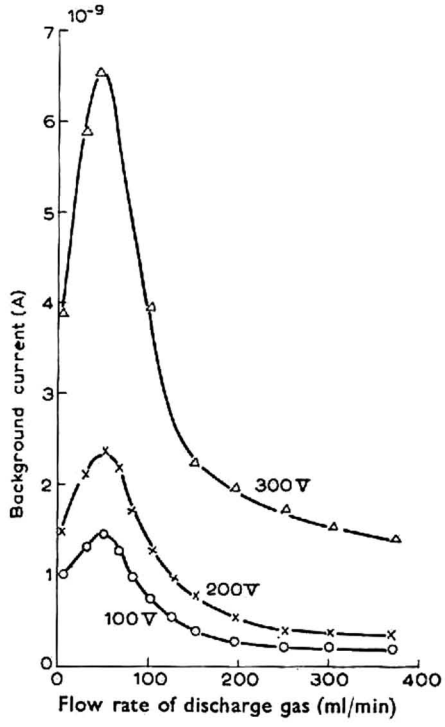


Fig. 3. Background current as a function of the flow rate of the discharge gas. Carrier gas = He 30 ml/min; discharge current = 30 μA; anode voltage = 100, 200, 300 V; impurity trap = activated charcoal (—196°); column = molecular sieve 13X 1.5 m (20°).

violet radiation that passes through the sensing region. The increase in the discharge gas flow dilutes the impurity and increases the radiation intensity. (2) The carrier gas is much more contaminated with impurities than the discharge gas, as it has passed through a sample port and a chromatographic column. At lower discharge gas flows, the carrier gas that has flowed in the sensing chamber can diffuse towards the discharge chamber counter to the discharge gas flow. The effect of this counter-flow diffusion is to reduce the intensity of ultraviolet radiation because of absorption by the impurities contained in the diffusing carrier gas. The increase in the discharge gas flow suppressed the counter-flow diffusion of the carrier gas and increases the radiation intensity.

The increase in the background current with the discharge gas flow continues until the dilution of the impurities in the sensing chamber becomes appreciable. Thereafter, further reduction of the concentration causes a lowering of ionization. The decrease in the background current with higher discharge gas flows is attributable to this effect.

Effect of the impurity traps

As would be expected from the results of the foregoing measurements of the background current, the output signal of the detector should be influenced by the impurities contained in both the discharge and the carrier gas. To study this effect, chromatograms were taken, setting the temperature of the impurity traps to different values and operating the detector under constant conditions.

Fig. 4 shows two typical chromatograms of 0.1 ml of coal gas. The impurity traps of the two gas flows were kept at room temperature (right chromatogram) and then

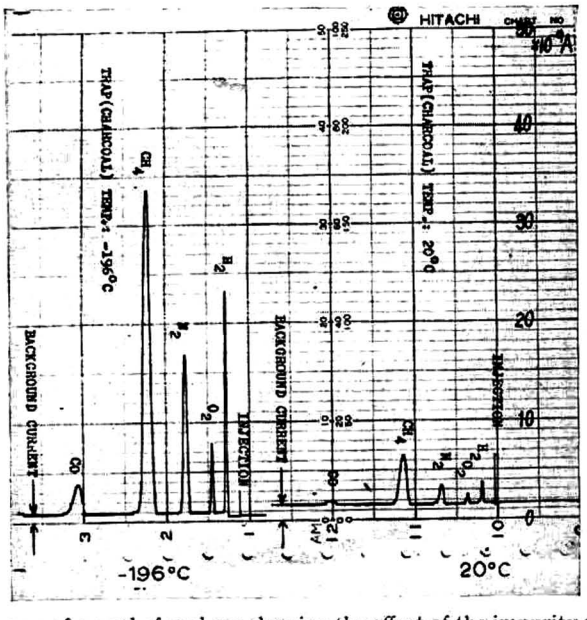


Fig. 4. Chromatograms of 0.1 ml of coal gas showing the effect of the impurity traps. Full scale = $50 \cdot 10^{-9}$ A. Carrier gas = He 30 ml/min; discharge gas = He 150 ml/min; discharge current = 60 μA; anode voltage = 100 V; column = molecular sieve 13X 1.5 m (20°).

cooled to liquid nitrogen temperature (left chromatogram). Other operational conditions were as follows:

Carrier gas	= He 30 ml/min.
Discharge gas	= He 150 ml/min.
Discharge current	= 60 μA.
Anode voltage	= 100 V.
Ion trap voltage	= 0 V.
Column	= Molecular sieve 13X 1.5 m (20°).

In both of these chromatograms, signal currents corresponding to hydrogen, oxygen, nitrogen, methane and carbon monoxide appear as a positive deflection. These ionization peaks can only come from the photoionization process, since it is not believed that ionization by collision of electrons could take place at an anode voltage of 100 V.

The effect of the gas treatment on the output signal is quite clearly shown in Fig. 4: corresponding ionization peaks in the two chromatograms differ by a factor of about 10. This difference can be attributed to the fact that, with the impurity traps kept at room temperature, permanent gases cannot be removed from the discharge gas. Such impurities absorb ultraviolet light and decrease the number of photons passing through the sensing chamber, so that ionization peaks must be diminished.

It is to be noted that the background current in the right chromatogram is about two times as great as that in the left chromatogram. This observation is consistent with the view that the background current is determined by the concentration of the impurities.

Owing to these observations, all measurements described below were conducted with the impurity traps immersed in liquid nitrogen, and it was found that, with proper precautions, results were reproducible for a long time period of operation.

Effect of the discharge gas flow

As indicated in the studies of the background current, the flow rate of the discharge gas is an important parameter for the satisfactory operation of the detector. To illustrate this effect, four representative chromatograms are shown in Fig. 5. These chromatograms were obtained with 0.1 ml of coal gas, varying the flow rate of the discharge gas and keeping other operational conditions constant.

These data reveal some interesting effects; the outstanding ones can be briefly summarized as follows.

(1) At 30 ml/min, inversions occur in the peaks of H_2, N_2 and CH_4.

(2) With increasing gas flow, these peak inversions become diminished; the inversion of the N_2 peak disappears at 70 ml/min and those of H_2 and CH_4 at 150 ml/min.

(3) The peak of O_2 gives a maximum current at 70 ml/min, while that of CO is diminished as the flow rate is increased.

In addition to this preliminary survey, measurements were also made for H_2, O_2 and N_2 to obtain more quantitative information on these phenomena. The results obtained are shown in Figs. 6, 7 and 8. In these figures, the dashed portions of the curves correspond to current values at which inversion occurs.

It can be seen from these figures that, with increasing gas flow, the peak current increases at first, reaches a maximum and then decreases. This is similar to the background current *vs.* gas flow characteristic.

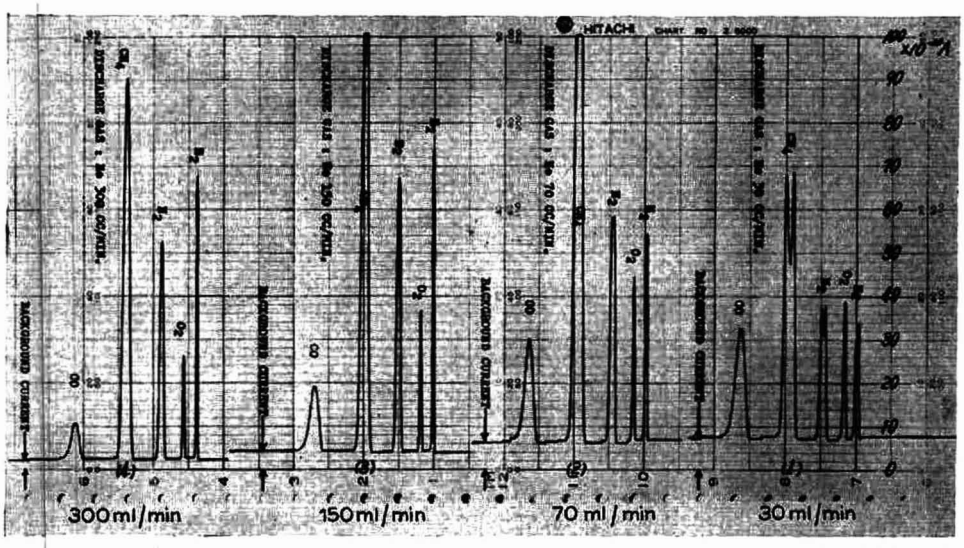


Fig. 5. Chromatograms of 0.1 ml of coal gas showing the effect of the discharge gas flow. Full scale = $100 \cdot 10^{-10}$ A. Carrier gas = He 30 ml/min; discharge current = 30 μA; anode voltage = 100 V; column = molecular sieve 13X 1.5 m (20°).

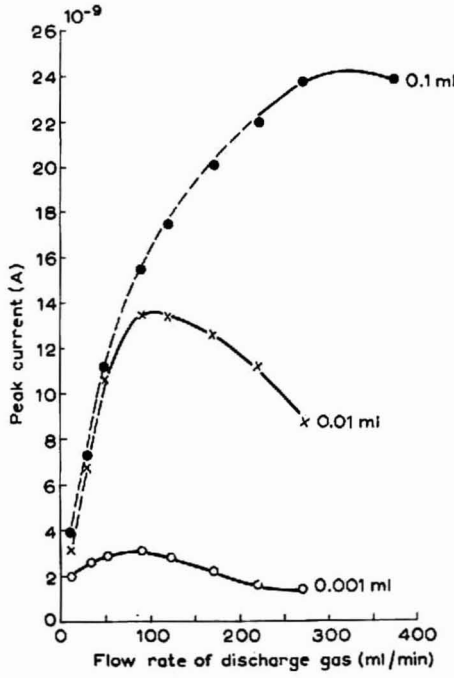


Fig. 6. Effect of the flow rate of the discharge gas on the peak current of H_2. Sample quantity = 0.1, 0.01, 0.001 ml; carrier gas = He 30 ml/min; discharge current = 30 μA; anode voltage = 100 V; column = molecular sieve 13X 1.5 m (20°).

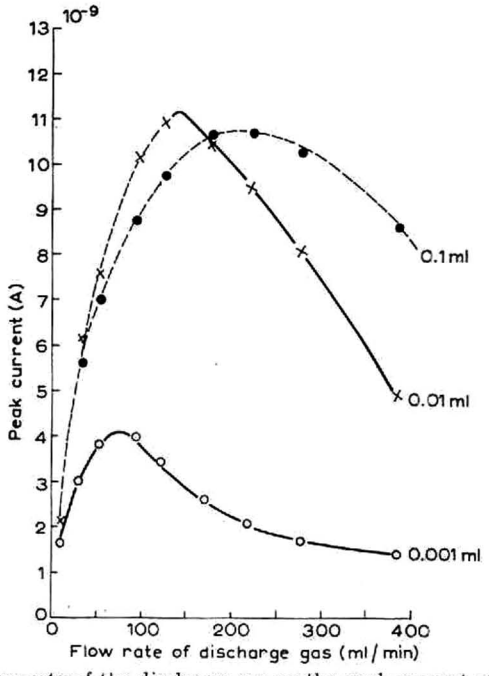


Fig. 7. Effect of the flow rate of the discharge gas on the peak current of O_2. Sample quantity = 0.1, 0.01, 0.001 ml; carrier gas = He 30 ml/min; discharge current = 30 μA; anode voltage = 100 V; column = molecular sieve 13X 1.5 m (20°).

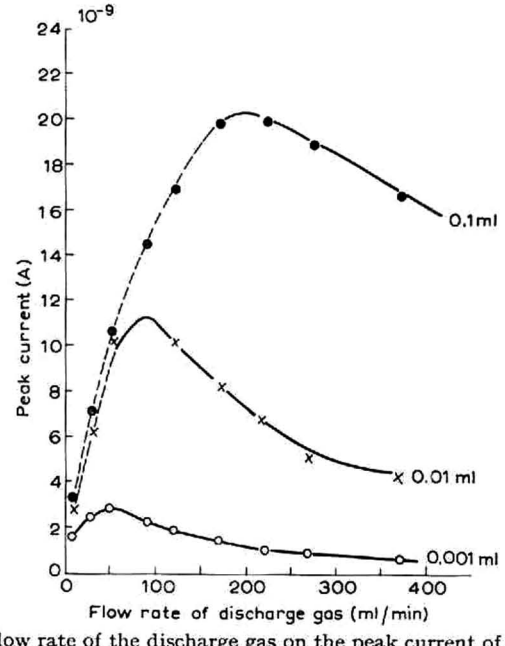


Fig. 8. Effect of the flow rate of the discharge gas on the peak current of N_2. Sample quantity = 0.1, 0.01, 0.001 ml; carrier gas = He 30 ml/min; discharge current = 30 μA; anode voltage = 100 V; column = molecular sieve 13X 1.5 m (20°).

With this similarity as a basis, we can interpret the experimental curves as follows. The discharge gas has two effects, that of increasing the number of photons that pass through the space between the sensing electrodes and that of reducing the concentration of the sample gas in the sensing chamber. The initial increase in the current at lower discharge gas flows is caused by the former effect. The latter effect is, of course, to reduce the ionization efficiency; this accounts for the slow decline in the region of higher discharge gas flows. These opposing effects should yield a maximum point on the current–gas flow curves.

The peak inversion that is likely to occur when the gas flow is low, and when the sample quantity is large, is attributed to the diffusion of the sample gas counter to the discharge gas flow. As the density of the sample gas in the sensing chamber is increased, the rate of the counter-flow diffusion increases, and fractions of the diffusing sample gas can move closer to the discharge chamber. Thus, with increasing density of the sample gas, the absorption of ionizing photons increases to a stage where the resulting ions cannot be collected by the ion collector, but are captured by the ion trap or recombine with electrons. This reduces the effective radiation intensity in the sensing region, resulting in a decrease in ion current at higher densities of the sample gas.

Effect of the anode voltage

Fig. 9 shows the peak current as a function of the anode voltage for 0.01 ml of H_2, O_2 and N_2. It can be seen that H_2 and N_2 yield a saturation current below *ca.* 300 V, while for O_2 the current increases, though slightly, as the anode voltage is increased.

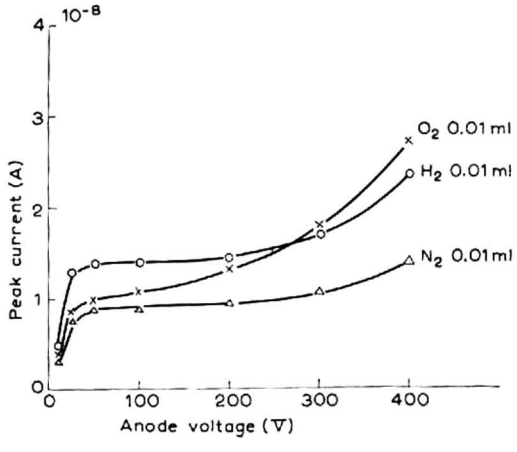


Fig. 9. Effect of the anode voltage on the peak current of H_2, O_2 and N_2. Sample quantity = 0.01 ml; carrier gas = He 30 ml/min; discharge gas = He 150 ml/min; discharge current = 30 μA; column = molecular sieve 13X 1.5 m (20°).

The signal to background ratio that can be obtained from Figs. 2 and 9 decreases with increasing anode voltage; the value for 0.01 ml of H_2, for instance, varies from *ca.* 45 at 100 V to *ca.* 3 at 400 *V*. Since increase in the background current is expected to increase the noise level, it is advisable to operate the detector at lower anode voltages.

Sensitivity and limit of detection

The relation between the output current and the sample quantity was determined for H_2, O_2 and N_2. These sensitivity studies were made with H_2–air mixtures of different compositions, which were introduced by means of a sample port of 0.1 ml.

Fig. 10 shows a series of chromatograms that covers the range of H_2 concentration from 1 to 0.1% (1–0.1 μl). Fig. 11 also shows two chromatograms of air–H_2

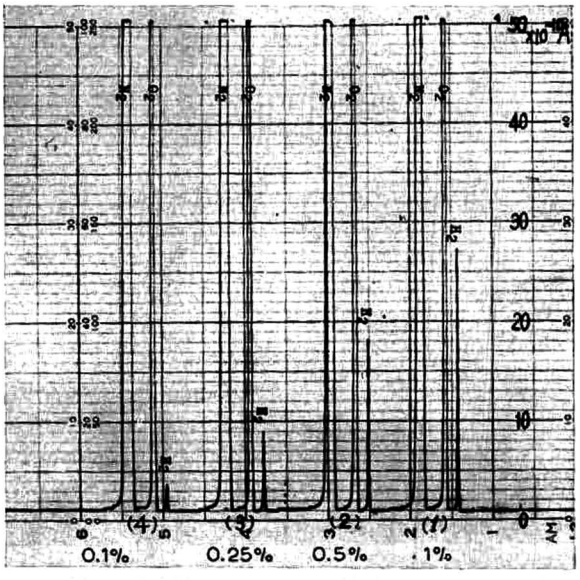


Fig. 10. Chromatograms of 0.1 ml of H_2-air mixtures. Full scale = $50 \cdot 10^{-10}$ A. (1) H_2 1%; (2) H_2 0.5%; (3) H_2 0.25%; (4) H_2 0.1%. Carrier gas = He 30 ml/min; discharge gas = He 150 ml/min; discharge current = 30μA; anode voltage = 100 V; column = molecular sieve 13X 1.5 m (20°).

(100:0.1) and air–H_2 (100:0.05) analogous to those in Fig. 10, except that the ordinate scale is expanded by a factor of 10. It can be seen that 0.1 μl of H_2 gives a peak current of $3.5 \cdot 10^{-10}$ A while the noise level was found to be about $3 \cdot 10^{-13}$ A. This indicates that the smallest detectable peak corresponds to approximately $1 \cdot 10^{-4}$ μl.

In Fig. 12 the sensitivity curves are shown, as plotted from the chromatograms obtained in this way. The values of the minimum detectable quantity for H_2, O_2 and N_2 were calculated and found to be $4.5 \cdot 10^{-12}$, $1.4 \cdot 10^{-11}$ and $1.6 \cdot 10^{-11}$ g/sec or in terms of concentration in the carrier gas $9 \cdot 10^{-12}$, $2.8 \cdot 10^{-11}$ and $3.2 \cdot 10^{-11}$ g/ml respectively.

It should be noted, however, that the sensitivity curves based on the peak current are not at 45°, indicating that the detector is non-linear. This may possibly be attributed to the particular cell geometry, and could be improved by modifying the design of the electrode system.

CONCLUSIONS

The results presented here show that the photoionization method, in which helium is used as both the discharge and the carrier gas, is suitable for the determination of minute quantities of inorganic gases. In the case of organic vapours, C_2–C_4 hydrocar-

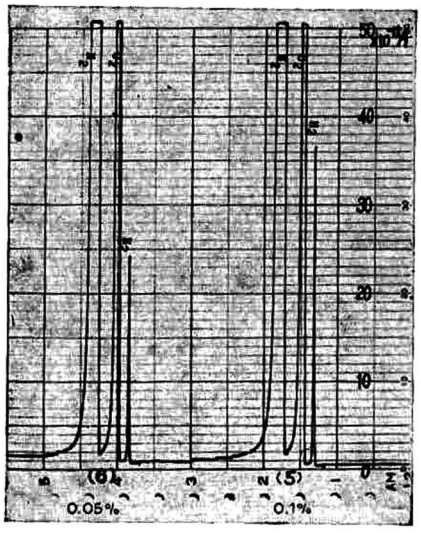


Fig. 11. Chromatograms of 0.1 ml of H_2–air mixtures. Full scale = $50 \cdot 10^{-11}$ A. (5) H_2 0.1%; (6) H_2 0.05%. Carrier gas = He 30 ml/min; discharge gas = He 150 ml/min; discharge current = 30 μA; anode voltage = 100 V; column = molecular sieve 13X 1.5 m (20°).

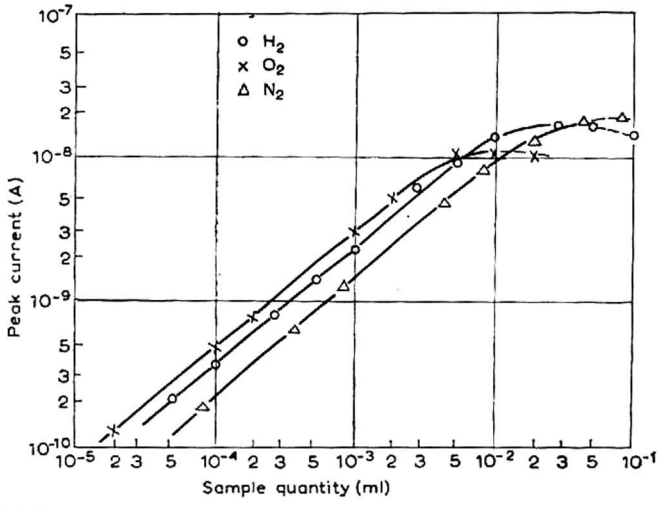


Fig. 12. Sensitivity curves for H_2, O_2 and N_2. Carrier gas = He 30 ml/min; discharge gas = He 150 ml/min; discharge current = 30 μA; anode voltage = 100 V; column = molecular sieve 13X 1.5 m (20°).

bon mixtures were tested and determined with satisfactory results, although this work is not reported in the present paper. These results depend on the fact that the helium discharge emits highly energetic ultraviolet radiation capable of ionizing molecules (with the exception of neon).

A most important point is the effect of impurities, which can absorb ultraviolet radiation. This gives rise to background current and, furthermore, reduces the ionization efficiency of the sample gas. An extreme degree of purification is therefore necessary to ensure satisfactory operation of the detector.

It is of interest to determine the concentration of the impurity encountered in our experiments. If we assume that the impurity was air, it is possible to estimate its concentration by injecting air of known quantity and relating the background current to the peak currents of the injected air. The concentration of the impurity in He in the sensing chamber is given by the formula:

$$C = \frac{I_B/u}{\{(I_{O_2} \times \sigma_{O_2}) + (I_{N_2} \times \sigma_{N_2})\}/W}$$

where C = concentration of the impurity.
I_B = background current (A).
u = sum of the flow rate of the carrier and the discharge gas (ml/sec).
W = quantity of the injected air (ml).
I_{O_2}, I_{N_2} = peak current of O_2 and N_2 of the injected air (A).
σ_{O_2}, σ_{N_2} = band width of O_2 and N_2 peak (sec).

In order to obtain a numerical answer from this equation, we use the measured values obtained under the conditions of the sensitivity experiment (Fig. 12): $I_B = 3 \cdot 10^{-10}$ A, $u = 3$ ml/sec, $W = 0.01$ ml, $I_{O_2} = 5.3 \cdot 10^{-9}$ A, $I_{N_2} = 8 \cdot 10^{-9}$ A, $\sigma_{O_2} = 11$ sec and $\sigma_{N_2} = 16$ sec. Substituting these values into the equation we get $C = 5.3 \cdot 10^{-6}$.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. I. MAKINO, Director of the Naka works, Hitachi Ltd. and Mr. S. TAKEI for their interest and encouragement during the course of this investigation.

He wishes to express his indebtedness to Mr. S. D. NOREM of the Perkin-Elmer Corporation for his helpful discussions and his kind linguistic help in completing the original manuscript. He also wishes to thank Mr. M. YAMAMOTO for his useful discussions and Mr. I. ASAKAWA for his assistance in the measurements.

SUMMARY

This report describes studies of the photoionization detector which has been developed for the measurement of small amounts of components in the gas chromatographic analysis. By the use of helium as both discharge and carrier gas it is found that the detector can respond to permanent gases with high detection sensitivity, particularly when the helium is purified by the impurity traps.

Details are given of the operating characteristics, and the effects of impurity on the background current and the output signal is discussed.

REFERENCES

[1] M. YAMANE, *J. Chromatog.*, 9 (1962) 162.
[2] M. YAMANE, *J. Chromatog.*, 11 (1963) 158.
[3] J. E. LOVELOCK, *J. Chromatog.*, 1 (1958) 35.
[4] J. E. LOVELOCK, *Nature*, 188 (1960) 401.
[5] J. J. HOPFIELD, *Astrophys. J.*, 72 (1930) 133.
[6] Y. TANAKA, A. S. JURSA AND F. J. LEBLANC, *J. Opt. Soc. Am.*, 48 (1958) 304.
[7] R. E. HUFFMAN, Y. TANAKA AND J. C. LARRABEE, *J. Opt. Soc. Am.*, 52 (1962) 851.
[8] R. E. HUFFMAN, Y. TANAKA, J. C. LARRABEE AND R. NOVACK, *Ionization Phenomena in Gases, II, Munich 1961*, North-Holland Publishing Co., Amsterdam, 1962, p. 1938.

COLLECTE D'ESTERS D'ACIDES GRAS MARQUÉS AU TRITIUM ET AU CARBONE-14 ÉLUÉS PAR CHROMATOGRAPHIE GAZ–LIQUIDE

JEAN BEZARD, PHILIPPE BOUCROT ET GUY CLEMENT

Laboratoire de Physiologie Animale, Faculté des Sciences de Dijon, Côte d'Or (France)

(Reçu le 29 juillet 1963)

INTRODUCTION

Dans les études métaboliques comportant des acides gras marqués, il est souvent indispensable de connaître la distribution de la radioactivité entre les acides gras d'un échantillon, ainsi que l'activité spécifique de chaque acide gras pris individuellement. Ceci peut être réalisé en principe de façon simple et précise par la mesure de l'activité des acides gras élués par chromatographie gaz–liquide.

Plusieurs méthodes ont déjà été décrites; elles se ramènent à deux types: soit le comptage direct, en continu, à la sortie de colonne[1,2], soit la collecte individuelle des esters d'acides gras et comptage ultérieur[3–7], seule technique possible lorsque l'activité d'un pic est trop faible pour être comptée valablement pendant la durée d'émergence du pic. Ces méthodes ont presque toutes été développées en employant des acides gras marqués au carbone-14.

Dans ce travail, nous avons comparé le comportement en chromatographie gaz–liquide d'esters d'acides gras marqués au carbone-14 ou au tritium, à la fois sur colonne d'apiezon et sur colonne de polyester DEGS. Nous avons fait une étude systématique de l'influence des différents facteur sur la collecte de ces esters, et nous nous sommes attachés surtout à l'étude des trainées radioactives.

TECHNIQUES

Chromatographie gaz–liquide

On utilise un appareil Barber-Colman modèle 10 avec détection par ionisation dans une microcellule au ^{90}Sr. Un séparateur (by-pass) placé à la sortie de la colonne, divise le courant d'argon en deux courants, un de récupération et l'autre de détection (Fig. 1). On emploie des colonnes de verre en U de 5 mm de diamètre intérieur, de 60 cm de longueur lorsque la phase stationnaire est l'apiezon, de 2 m lorsque c'est le succinate de diethylène glycol (DEGS). Le support inerte, du chromosorb W 80–100 mesh (175–147 μ) est imprégné de liquide stationnaire selon la méthode préconisée par HORNING[8] dans la proportion de 15–20 g d'apiezon ou de 25–30 g de DEGS pour 100 g de support. Pour une température de 170–180° et sous une pression d'entrée d'argon de 1.5 kg/cm^2, on obtient dans ces conditions une bonne résolution de la séparation des différents acides gras, avec un temps de rétention d'environ 20 min pour le stéarate de méthyle.

Nous avons retenu les conditions expérimentales suivantes:

Taux de séparation du courant d'argon de sortie: 95.5 % en récupération et 4.5 % en détection.

Tension appliquée au détecteur: 1250 V.

Contre-balayage d'argon dans la cellule de détection: 100 ml/min.

Température de la colonne: 170°.

Température de la cellule: 200°.

On traduit les aires enregistrées en microgrammes d'acides gras, aux différentes sensibilités de l'enregistreur, pour le calcul des activités spécifiques absolues. Dans ces conditions, on peut évaluer avec précision des quantités d'acide stéarique allant de 0.7 à 700 μg.

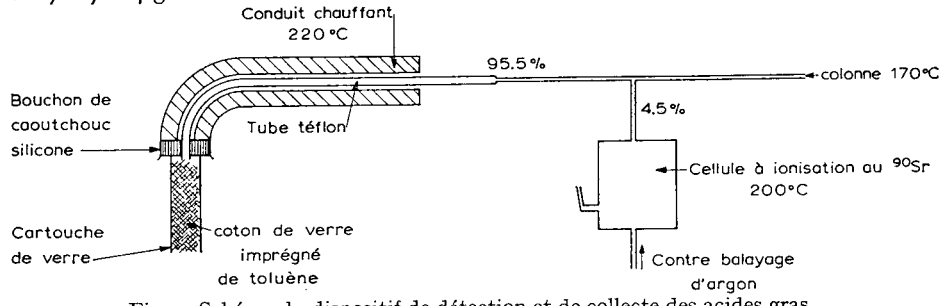


Fig. 1. Schéma du dispositif de détection et de collecte des acides gras.

Collecte des esters d'acides gras

Elle s'effectue par une méthode voisine de celle décrite par MEINERTZ ET DOLE[6]. A l'émergence de la colonne, les vapeurs d'esters méthyliques passent à travers un tube de téflon de 1.5 mm de diamètre intérieur et de 20 cm de longueur entouré d'un manchon chauffant (température 220°) fermé par une rondelle de caoutchouc siliconé de 1 cm d'épaisseur (voir Fig. 1).

Pour collecter les vapeurs, on applique contre le caoutchouc siliconé des cartouches de verre de 3.5 cm de longueur et de 6 mm de diamètre intérieur, ouvertes aux deux extrémités. Ces cartouches contiennent de la laine de verre imprégnée de toluène sur laquelle se condensent les vapeurs. On effectue le changement des cartouches manuellement, en se repérant sur le chromatogramme qui est enregistré simultanément.

On introduit la laine de verre dans un pot à scintillation en la poussant à l'aide d'un agitateur, puis on rince les parois intérieures de la cartouche avec 20 ml de liquide scintillant qu'on recueille dans le pot. On prend soin d'amener la laine de verre sur le fond du récipient afin qu'elle ne fasse pas écran devant la fenêtre des photomultiplicateurs; on élimine aussi les bulles d'air emprisonnées en pressant la laine de verre avec l'agitateur, ce qui facilite par là même la mise en solution des esters condensés; les fragments de fibre de verre disséminés dans le liquide décantent rapidement.

Afin d'obtenir une récupération aussi quantitative que possible, nous avons préféré transférer la laine de verre dans le pot plutôt qu'entraîner les esters par rinçage comme le font MEINERTZ ET DOLE[6].

Mesure de l'activité

Elle se fait dans un appareil Tracerlab à scintillation liquide modèle LSC 10. Le liquide scintillant contient, par litre, 4 g de PPO (2,5-diphényloxazole), 100 mg de

POPOP (1,4-bis-2-(5-phényloxazolyl)-benzène) et on prend comme solvant du toluène Merck (pour analyses et chromatographie)

Les conditions de comptage offrant le meilleur rendement, comptetenu du bruit de fond sont les suivantes:

Tension appliquée aux photomultiplicateurs: 1600 V pour le tritium et 1300 V pour le carbone-14.

Comptage des impulsions en coïncidence dans les deux photomultiplicateurs, sans rejet des impulsions de haute énergie (2 channels) et gain de 24, pour les deux isotopes.

Dans ces conditions, le rendement pour le tritium est de 15 % avec un bruit de fond de 88 cpm (laine présente dans le liquide scintillant). Pour le carbone-14, le rendement est de 60 % et le bruit de fond de 82 cpm.

Préparation des échantillons et conduite des expériences

Dans nos expériences, on utilise du methyl-stéarate-1-^{14}C et -9,10-^{3}H. Les échantillons sont tout d'abord purifiés par chromatographie gaz-liquide sur colonne DEGS après dilution avec du stéarate inactif; on recueille uniquement la fraction correspondant à la partie la plus étroite du pic. On s'est ensuite assuré de leur pureté en les rechromatographiant sur colonne DEGS.

Afin de se trouver dans des conditions de charge de colonne aussi comparables que possible, on choisit les activités spécifiques inversement proportionnelles au rendement du comptage. On compte ainsi un même nombre de coups pour une même quantité injectée, que le traceur soit du tritium ou du carbone-14.

Les colonnes utilisées, apiezon et DEGS, dont on a donné plus haut les caractéristiques, sont des colonnes neuves, qui n'ont encore reçu aucune injection d'acides gras radioactifs; des essais de vieillissement de colonne ont été effectués spécialement.

La durée de collecte du pic actif a été prise égale à $^{1}/_{8}$ du temps de rétention. Cette durée, légèrement différente sur les deux types de colonne, mais voisine de 3.3 min, correspond à une zone de collecte sur le chromatogramme comprenant la totalité du pic d'élution; elle a été choisie comme unité de temps de collecte dans l'étude systématique de l'activité retenue sur la colonne. Nous recueillons en une seule fraction, soit pendant 5.5 unités de temps, tout ce qui est élué avant le pic 18:0. Nous collectons ensuite le pic actif en une seule fraction (prise comme unité), puis nous collectons 9 fractions successives de 1 unité de temps, 3 fractions de 2 unités et 3 fractions de 3 unités, soit une évaluation de la radioactivité contaminante pendant environ 80 min après l'élution du pic actif. Les activités collectées sont exprimées en cpm et en pour cent de l'activité totale récupérable qui représente 95 % de l'activité injectée.

RÉSULTATS

1. Pic unique de méthyl-stéarate-1-^{14}C ou -9,10-^{3}H (Tableau I)

(*a*) *Fraction collectée avant le pic 18:0.* Sur colonne DEGS, aucune radioactivité n'apparaît dans cette fraction, tant pour les acides gras marqués au tritium que pour ceux marqués au carbone-14. Sur colonne d'apiezon, elle est extrêmement faible: 0.10 et 0.13 % respectivement de l'activité récupérable pendant les 5.5 unités de temps.

(*b*) *Fraction collectée pendant l'élution du pic 18:0.* L'activité mesurée sur cette fraction représente plus de 95 % de l'activité totale récupérable, dans toutes les

expériences. Ce pourcentage est d'ailleurs sensiblement constant quelle que soit la phase stationnaire utilisée (apiezon, DEGS) et quel que soit l'acide gras marqué injecté.

(*c*) *Fractions collectées après le pic 18:0.* On détecte de la radioactivité pendant 80 min après élution du stéarate: pendant la première unité de temps, elle représente 0.6 à 1.0 % de l'activité totale récupérable sur colonne d'apiezon et 1.2 à 1.6 % sur colonne de DEGS. L'activité décroît ensuite régulièrement: 0.10 à 0.15 % à la sixième unité, soit 20 min environ après élution du pic radioactif; après 1 h, elle n'est plus que de 0.01 à 0.02 %.

Si l'on compare la décroissance de la radioactivité contaminante, il ne semble pas qu'il y ait de différence selon le type d'acide marqué. On voit sur le graphique

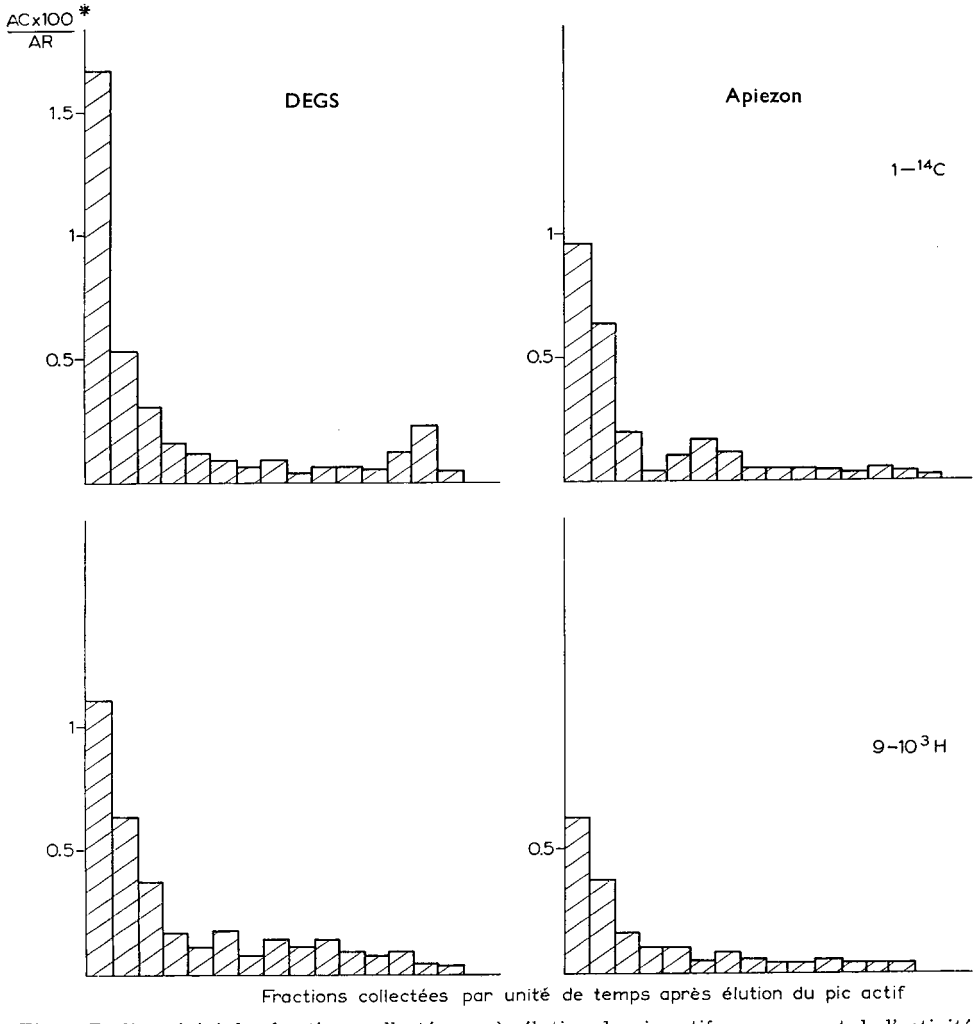


Fig. 2. Radioactivité des fractions collectées après élution du pic actif en pour cent de l'activité récupérable. Injection de méthyl-stéarate-1-^{14}C et -9,10-^{3}H sur colonne apiezon et DEGS (voir notes Tableau I).

TABLEAU I

RADIOACTIVITÉ DES FRACTIONS COLLECTÉES APRÈS INJECTION DE MÉTHYL-STÉARATE-1-^{14}C ET -9,10-^{3}H SUR COLONNES APIEZON ET DEGS

Fractions collectées	*Nombre d'unités de temps de collecte**	*Radioactivité** des fractions sur*									
		Apiezon				*DEGS*					
		1-^{14}C AR = 39,000 cpm		*9,10-^{3}H AR = 39,500 cpm*		*1-^{14}C AR = 36,000 cpm*		*9,10-^{3}H AR = 36,500 cpm*		*9,10-^{3}H AR = 97,500 cpm*	
		AC (cpm)	*AC × 100/AR (%)*	*AC (cpm)*	*AC × 100/AR (%)*	*AC (cpm)*	*AC × 100/AR (%)*	*AC (cpm)*	*AC × 100/AR (%)*	*AC (cpm)*	*AC × 100/AR (%)*
Avant 18:0	5.5	43 ± 3***	0.10	51 ± 4	0.13	0	0	0	0	0	0
Pic 18:0	1	38,504 ± 62	98.7	38,080 ± 62	96.4	34,435 ± 58	95.6	35,824 ± 60	98.1	93,312 ± 97	95.7
Après 18:0	1	375 ± 7	0.96	244 ± 6	0.62	605 ± 8	1.68	423 ± 7	1.16	1643 ± 13	1.69
	1	248 ± 6	0.64	148 ± 5	0.37	192 ± 5	0.53	230 ± 5	0.63	541 ± 8	0.55
	1	76 ± 4	0.20	60 ± 4	0.15	112 ± 4	0.31	134 ± 5	0.37	285 ± 6	0.29
	1	17 ± 3	0.04	34 ± 3	0.09	57 ± 3	0.16	59 ± 4	0.16	220 ± 6	0.23
	1	41 ± 3	0.10	33 ± 3	0.09	44 ± 3	0.12	35 ± 3	0.10	168 ± 5	0.17
	1	66 ± 4	0.17	18 ± 3	0.04	34 ± 3	0.09	63 ± 4	0.17	97 ± 4	0.10
	1	46 ± 3	0.12	26 ± 3	0.07	22 ± 3	0.06	22 ± 3	0.06	86 ± 4	0.09
	1	21 ± 3	0.05	14 ± 3	0.04	32 ± 3	0.09	46 ± 4	0.13	68 ± 4	0.07
	1	20 ± 3	0.05	11 ± 3	0.03	14 ± 3	0.04	38 ± 3	0.10	54 ± 4	0.06
	2	19 ± 3	0.05	13 ± 3	0.03	22 ± 3	0.06	47 ± 4	0.13	33 ± 1	0.03
	2	17 ± 3	0.04	19 ± 3	0.04	21 ± 3	0.06	28 ± 3	0.08	68 ± 4	0.07
	2	10 ± 3	0.03	12 ± 3	0.03	20 ± 3	0.05	20 ± 3	0.05	40 ± 4	0.04
	3	20 ± 3	0.05	11 ± 3	0.03	44 ± 3	0.12	29 ± 3	0.08	21 ± 3	0.02
	3	16 ± 3	0.04	12 ± 3	0.03	84 ± 4	0.23	12 ± 3	0.03	56 ± 4	0.06
	3	8 ± 3	0.02	0	0	19 ± 3	0.05	7 ± 3	0.02	19 ± 2	0.02
	Totaux	39,547	101.36	38,786	98.19	35,757	99.25	37,017	101.37	96,711	99.19

* 1 unité = 3.3 min = $^{1}/_{6}$ du temps de rétention.

** AR = activité récupérable, égale aux 95.5% de l'activité injectée, le système de "by-pass" prélevant 4.5% pour la détection; AC = activité collectée par fraction.

*** ± écart type.

(Fig. 2) que la décroissance a même allure, par contre elle diffère légèrement selon le type de colonne apiezon, ou DEGS.

Lorsqu'on injecte une quantité plus grande d'ester (Tableau I, 4 dernières colonnes), l'activité contaminante est relativement plus importante dans l'unité de temps qui suit immédiatement l'élution du pic: 1.76 % pour 93,300 cpm au lieu de 1.18 % pour 35,800 cpm; mais il n'y a pas de proportionnalité: pour une charge 2.6 fois plus importante, le pourcentage d'activité est multiplié seulement par 1.5. Ensuite, l'activité décroît. Dès la deuxième unité de temps on atteint un même niveau, que la charge soit forte ou faible (Fig. 3).

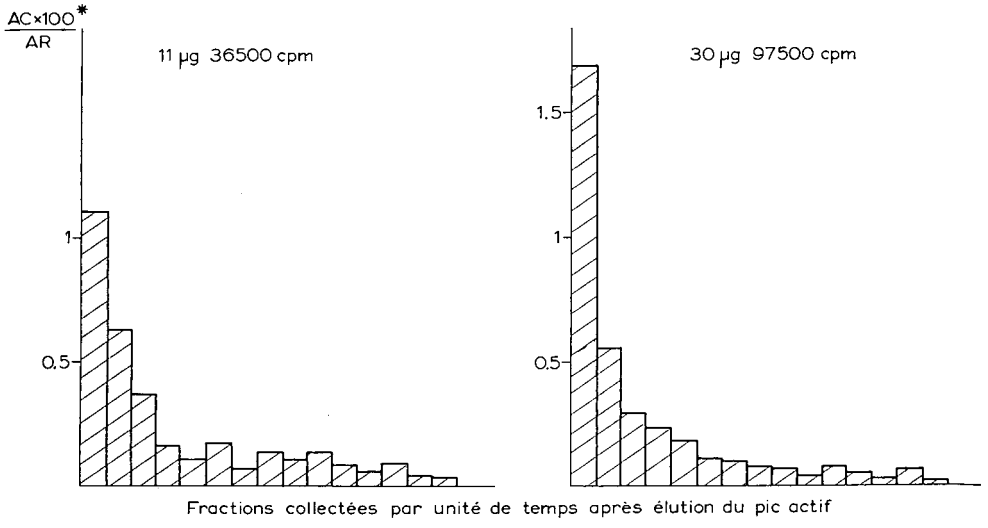


Fig. 3. Radioactivité des fractions collectées après élution du pic actif en pour cent de l'activité récupérable. Injection de deux charges différentes de méthyl-stéarate-9,10-^{3}H sur colonne DEGS (voir notes Tableau I).

2. Addition de méthyl-stéarate non radioactif

Pour voir si la traînée constatée après élution d'un pic unique radioactif est affectée par l'élution consécutive de pics non actifs, nous avons fait 5 injections consécutives de méthyl-stéarate tritié séparées à intervalles réguliers égaux à une unité de temps de collecte en commençant aussitôt après l'injection du pic 18:0 tritié (Tableau II).

Si l'on compare ces résultats avec ceux du Tableau I, on voit que l'élution d'esters d'acides gras inactifs ne modifie absolument pas l'allure de la traînée contaminante laissée par un ester gras radioactif. Le fait est valable pour les deux catégories de phases stationnaires.

3. Effet de la durée d'utilisation de la colonne

Nous n'avons fait qu'un seul essai sur colonne DEGS en pratiquant des collectes sur une colonne neuve, puis sur la même colonne après 8 et 21 jours d'utilisation constante. Chaque fois, on pratique une injection d'une même quantité de méthyl-stéarate tritié: 11 µg, 36,500 cpm.

Nous avons représenté sur la Fig. 4, en pour cent de l'activité récupérable, la radioactivité par unité de temps, après élution du pic actif.

On voit que les résultats sont pratiquement identiques; il n'y a aucune augmentation des trainées radioactives du fait de l'utilisation de la colonne pendant la période testée.

TABLEAU II

RADIOACTIVITÉ DES FRACTIONS COLLECTÉES APRÈS INJECTION DE MÉTHYL-STÉARATE-9,10^{3}H, SUIVIE DE 5 INJECTIONS DE STÉARATE INACTIF

Fractions collectées	*Apiezon*		*DEGS*	
	Activité récupérable (AR) = 37,500 cpm		*Activité récupérable (AR) = 76,000 cpm*	
	Activité collectée (AC) (cpm)	*AC × 100/AR (%)*	*Activité collectée (AC) (cpm)*	*AC × 100/AR (%)*
Pic (radioactif) 18:0	36,355 ± 60	96.95	72,800 ± 90	95.79
Pics (inactifs) 18:0	237 ± 6	0.63	1300 ± 12	1.71
	149 ± 5	0.40	423 ± 7	0.56
	91 ± 4	0.24	302 ± 6	0.40
	59 ± 4	0.16	250 ± 6	0.33
	37 ± 3	0.10	192 ± 5	0.25

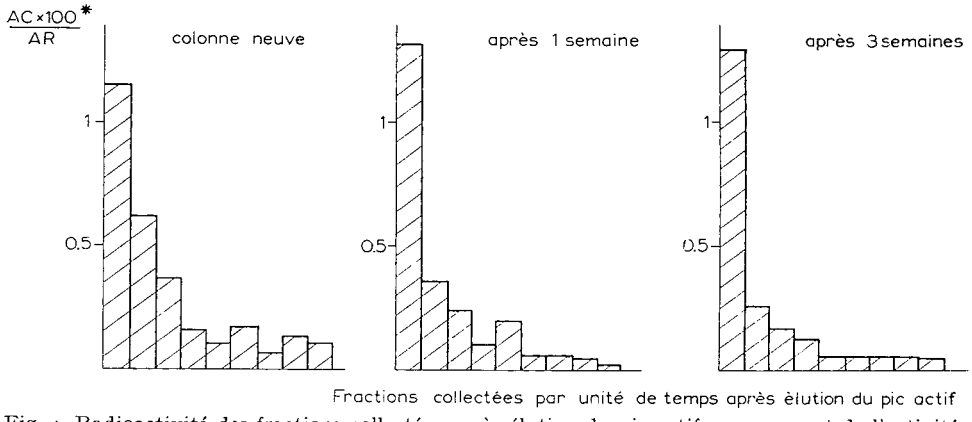


Fig. 4. Radioactivité des fractions collectées après élution du pic actif en pour cent de l'activité récupérable. Injection de méthyl-stéarate-9,10-^{3}H sur colonne DEGS neuve, après 1 semaine et après 3 semaines) (voir notes Tableau I).

4. Fractionnement d'un mélange de méthyl-stéarate et de méthyl-oléate marqués 1-^{14}C

Après injection du mélange d'esters radioactifs, nous avons recueilli 11 et 13 sous-fractions pour les pics 18:0 et 18:1 respectivement pour mesurer la radioactivité et nous avons fait figurer sur le chromatogramme d'élution (Fig. 5) le tracé correspondant à la radioactivité des sous-fractions (radiogramme). On voit que pour les doses injectées, on obtient une excellente résolution tant chromatographique que radiographique. Le sommet de la courbe d'activité précède légèrement celui du pic d'élution chromatographique. Ceci est dû simplement au fait que les circuits de détection et de récupération sont indépendants, ce dernier étant légèrement plus court. Cela mis à part, on note une excellente concordance entre les deux tracés.

Notons que les trainées radioactives n'apparaissent pas sur ce graphique, les unités de temps (sous-fractions) étant seulement de 24 sec au lieu de 3.3 min pour les expériences précédentes.

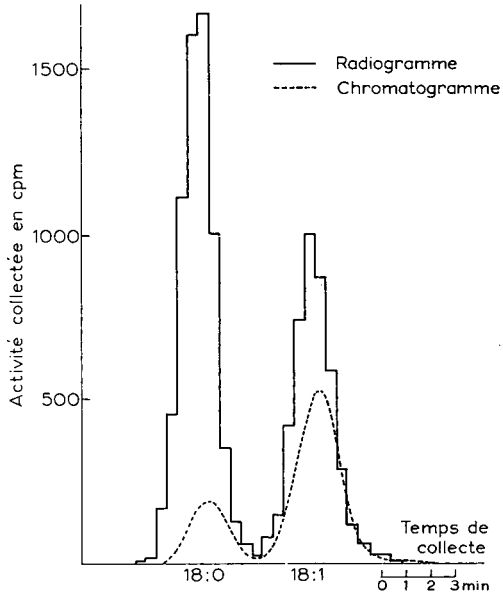


Fig. 5. Collecte du méthyl-stéarate et du méthyl-oléate-1-^{14}C après élution sur colonne DEGS: chromatogramme et radiogramme.

DISCUSSION ET CONCLUSIONS PRATIQUES

Des résultats antérieurs aux nôtres avaient déjà établi un certain nombre de faits relatifs à la collecte individuelle d'esters d'acides gras radioactifs élués par chromatographie gaz-liquide. On savait déjà par des études faites à l'aide de chromatographie et de systèmes de collecte de types divers[4-6] qu'on pouvait recueillir plus de 90 % de l'activité injectée dans le cas d'esters méthyliques d'acides gras marqués 1-^{14}C.

Dans le présent travail, nous montrons qu'il en est de même pour des esters d'acides gras marqués au tritium; notre pourcentage de récupération atteint presque 100 %. L'étude systématique que nous avons faite des trainées radioactives dans les conditions standard d'une analyse rapide sur microquantités (telle qu'elle peut être réalisée sur apparail Barber Colman à détection et récupération indépendantes) a permis d'établir un certain nombre de faits:

(i) Nous avons constaté que, toutes choses égales d'ailleurs (charges, activités, colonnes, etc.), les résultats sont identiques qu'il s'agisse d'acides gras marqués au tritium ou au carbone-14.

(ii) Par contre des différences apparaissent notamment dans l'importance et l'allure de la traînée radioactive selon la phase stationnaire utilisée: apiezon ou polyesters.

Une charge plus importante (activité spécifique égale) augmente l'activité retenue, surtout dans la première unité de temps. Le fait, déjà signalé par PASCAUD[7] pour le méthyl-palmitate-1-^{14}C est ici retrouvé avec le méthyl-stéarate-9,10-^{3}H.

Ces deux observations montrent que les traînées radioactives au voisinage immédiat du pic actif sont directement en rapport avec la descente plus ou moins rapide du pic et représentent donc essentiellement des traînées de pic: sur colonne d'apiezon en effet, la descente du pic est plus rapide que sur colonne DEGS et, lorsqu'on augmente la quantité d'ester injectée, la largeur du pic augmente en même temps que le sommet se trouve légèrement retardé, si bien qu'en se plaçant dans les mêmes conditions de collecte, on recueille dans l'unité de temps qui suit immédiatement une partie plus importante de la traînée.

Des esters inactifs élués immédiatement après un pic de méthyl-stéarate-9,10-^{3}H ne modifient absolument pas l'allure de la traînée radioactive laissée par le pic actif. MEINERTZ ET DOLE[6] l'avaient déjà signalé pour le méthyl-palmitate-1-^{14}C. Nous retrouvons la même chose avec le méthyl-stéarate tritié et cela quelle que soit la phase stationnaire utilisée. A cet égard, le fait qu'il n'y ait aucune différence entre une phase apolaire et une phase polyester n'est pas en faveur d'une intervention importante des phénomènes de transestérification à l'origine de la traînée radioactive comme on l'a suggéré[7].

Il est certain qu'il subsiste encore des traces de radioactivité longtemps après élution d'un pic actif. Peut-être s'agit-il d'une rétention d'esters sur la phase ou le support, suivie de réémission; quoi qu'il en soit, les contaminations par ces traînées radioactives sont, malgré tout, très minimes puisqu'elles s'abaissent déjà à 0.1 % environ dès la 4ème ou 5ème unité de temps après élution du pic actif pour diminuer progressivement ensuite. Quant à la radioactivité collectée avant le pic actif, elle est pratiquement inexistante lorsqu'il s'agit d'un ester déjà purifié par chromatographie gaz–liquide.

Nous terminerons par quelques conclusions pratiques. Nous dirons tout d'abord que les acides gras tritiés conviennent tout aussi bien que les acides gras marqués au carbone-14 pour les expériences de collecte et de mesure d'activité des fractions éluées.

Lorsqu'il s'agit de deux esters fortement radioactifs, même élués consécutivement, la séparation est aussi bonne au point de vue masse que radioactivité (Fig. 5). L'activité spécifique est très facile à déterminer puisqu'on connaît l'activité totale du pic et l'aire enregistrée simultanément. La faible activité d'un ester ne pose pas de problème en soi puisqu'on peut répéter la collecte, augmenter la quantité d'esters injectée et effectuer la mesure de la radioactivité pendant un temps assez long pour obtenir des résultats statistiquement valables. Mais une difficulté se présente lorsque des esters de faible activité sont élués très tôt après les esters fortement actifs. Dans ce cas, il est indispensable de connaître l'importance de la traînée radioactive au moment d'élution de l'ester.

Des graphiques tels que ceux que nous produisons dans ce travail montrent que l'on peut aisément décider de la validité des résultats.

Enfin, il n'est pas sans intérêt de constater qu'il n'y a aucune augmentation des traînées radioactives lorsqu'une colonne a servi pendant trois semaines d'une façon ininterrompue; il est vraisemblable qu'il en est ainsi aussi longtemps que son pouvoir de résolution n'est pas modifié. On peut donc presque recommander d'utiliser les co-

lonnes pour chromatographie de matériel alternativement actif et inactif, les traces d'activité résiduelle se trouvant ainsi éliminées entre temps.

RÉSUMÉ

Les esters d'acides gras marqués au tritium peuvent être collectés après élution par chromatographie gaz–liquide dans les mêmes conditions que les esters d'acides gras 1-^{14}C. L'étude comparée des traînées radioactives effectuée dans différentes conditions, donnent les mêmes résultats pour l'un et l'autre type d'acide gras marqué. On ne note pas de modifications dans les traînées radioactives en rapport avec la durée d'utilisation d'une colonne tant que son pouvoir de résolution est intact.

SUMMARY

Fatty acid esters labelled with tritium can be collected after gas–liquid chromatography under the same conditions as esters labelled with ^{14}C at C–1. Comparison of the radioactive tracings obtained under different conditions showed that the results were the same for both types of labelled fatty acid. The time during which a column is used has no effect, as long as the power of resolution remains intact.

BIBLIOGRAPHIE

1 G. POPJAK, A. E. LOWE, D. MOORE, L. BROWN ET F. A. SMITH, *J. Lipid Res.*, 1 (1959) 29.
2 A. T. JAMES ET E. A. PIPER, *J. Chromatog.*, 5 (1961) 265.
3 L. H. MASON, H. J. DUTTON ET L. R. BAIR, *J. Chromatog.*, 2 (1959) 322.
4 A. KARMEN, L. GIUFFRIDA ET R. L. BOWMAN, *J. Lipid Res.*, 3 (1962) 44.
5 A. K. HAJRA ET N. S. RADIN, *J. Lipid Res.*, 3 (1962) 131.
6 M. MEINERTZ ET V. P. DOLE, *J. Lipid Res.*, 3 (1962) 140.
7 M. PASCAUD, *J. Chromatog.*, 10 (1963) 125.
8 E. C. HORNING, E. A. MOSCATELLI ET C. C. SWEELEY, *Chem. Ind. (London)*, (1959) 751.

ÉTUDE DE LA VARIATION DU FACTEUR DE RÉPONSE DES CATHAROMÈTRES AVEC LE DÉBIT DU GAZ VECTEUR

GEORGES GUIOCHON

Laboratoire du Professeur L. Jacqué, École Polytechnique, Paris (France)

(Reçu le 30 août 1963)

Depuis fort longtemps, le catharomètre est utilisé comme détecteur à la sortie des colonnes de chromatographie en phase gazeuse et, malgré l'apparition récente des détecteurs à ionisation, en particulier du détecteur à ionisation de flamme, il ne semble pas perdre beaucoup de terrain.

Il est d'autant plus étonnant dans ces conditions de ne trouver que peu de renseignements sur l'influence du débit de gaz vecteur sur la réponse du catharomètre. La plupart des auteurs se sont en effet intéressés à l'influence du débit sur la ligne de base et, par conséquent, sur la stabilité du détecteur. On ne trouve que quelques indications contradictoires, mais aucune étude systématique de l'influence du débit sur la réponse quantitative.

Le travail de DIMBAT, PORTER ET STROSS[1] sur la sensibilité des catharomètres et, en particulier, l'expression de leur facteur de sensibilité:

$$S = \frac{A \cdot D \times C_1 \cdot C_2}{m} \qquad (1)$$

où A = l'aire du pic,
D = le débit,
C_1 = la sensibilité en mV/cm de l'enregistreur,
C_2 = l'inverse de la vitesse de déroulement du papier,
m = la masse de l'échantillon injectée,

sous-entend bien que l'aire du pic doit être inversement proportionnelle au débit pour que le facteur de sensibilité soit indépendant du débit, mais ce n'est pas explicitement formulé, encore moins démontré et aucune preuve expérimentale n'en est apportée[1]. Plus récemment KEULEMANS, dans son étude sur le facteur de réponse des cathatomètres, sous-entend de même qu'il est inversement proportionnel au débit, sans l'affirmer ni le démontrer[2].

Nous avons été amenés à étudier l'influence du débit sur la réponse des catharomètres à la suite de travaux d'analyse quantitative, pour rechercher les paramètres responsables des fluctuations de réponse et des erreurs de mesure. Nous allons nomtrer que l'aire d'un pic est inversement proportionnelle au débit, non seulement dans le cas des catharomètres mais dans celui de tous les détecteurs répondant à la concentration de soluté dans le gaz vecteur. Ceci résulte directement de ce que l'aire du pic mesurée sur l'enregistrement est l'intégrale par rapport au temps du signal fourni par le détecteur, puisque la vitesse de déroulement du papier est constante, tandis

que la masse de soluté est l'intégrale de sa concentration dans le gaz vecteur par rapport au volume de gaz débité. L'aire du pic serait constante si l'on effectuait un changement d'unités d'abscisses pour le graduer en volume. Comme l'axe des abscisses est toujours gradué en temps, l'aire mesurée est inversement proportionnelle au débit. On peut aussi démontrer cette relation par des considérations basées sur la théorie des plateaux, qui permettent d'obtenir également une relation simple entre la hauteur du pic obtenu et l'efficacité de la colonne. Ces résultats théoriques ont été vérifiés expérimentalement.

THÉORIE

Si l'on introduit une masse m d'un échantillon gazeux dans le premier plateau d'une colonne de chromatographie, supposée comporter N plateaux, la concentration du soluté dans chacun des plateaux, après passage d'un volume $n\delta v$ de phase mobile, dans des conditions telles que l'équilibre soit atteint après chaque passage du volume δv, est donnée par l'expression[3-5]:

$$W(n,r) = m \frac{n!}{r!(n-r)!} \left(\frac{\delta v}{V}\right)^r \left(1 - \frac{\delta v}{V}\right)^{n-r} \tag{2}$$

$$V = v_G + K v_L$$

où $W(n,r)$ = la quantité de soluté se trouvant dans le plateau r,
V = le volume de rétention par plateau théorique:

$$V = v^\circ_R = \frac{V^\circ_R}{N}$$

v_G et v_L sont les volumes de phases gazeuse et liquide contenues dans un plateau théorique:

$$v_G = \frac{V_G}{N}, \quad v_L = \frac{V_L}{N}$$

V_G et V_L étant les volumes de phases gazeuse et liquide contenues dans la colonne,
K = le coefficient de partage du soluté.

On peut montrer que si N est grand, et n tend vers l'infini (corrélativement δV tend vers zéro, de sorte que $n\delta V$ reste fini), la répartition ci-dessus est équivalente à une répartition gaussienne:

$$W(r) = \frac{m}{\sqrt{2\pi}\cdot\sigma(r)} \exp -\frac{(r-N)^2}{2\sigma^2(r)} \tag{3}$$

où $\sigma(r)$ = la déviation standard de la zone exprimée en nombre de plateaux. Dans ces conditions, la répartition de concentration est donnée par:

$$C(V) = \frac{m}{\sqrt{2\pi}\cdot\sigma(V)} \exp -\frac{(V-V_R^\circ)^2}{2\sigma^2(V)} \tag{4}$$

les concentrations étant exprimées en g/cm³. La déviation standard $\sigma(V)$ est reliée à N par l'expression:

$$N = \left(\frac{V_R^\circ}{\sigma(V)}\right)^2 \text{ où } \sigma(V) = \frac{V_R^\circ}{\sqrt{N}}$$

D'où:

$$C(V) = \frac{m\sqrt{N}}{\sqrt{2\pi}\cdot V_R^\circ} \exp - \frac{N(V - V_R^\circ)^2}{2(V_R^\circ)^2} \tag{5}$$

Cette expression montre que, pour un soluté et une colonne donnée, à température constante, la concentration maximum, correspondant au sommet du pic, qui est donnée par:

$$V = V_R^\circ$$

$$C(V)_{\max} = \frac{m\sqrt{N}}{\sqrt{2\pi}\cdot V_R^\circ} \tag{6}$$

sera d'autant plus grande que le nombre de plateaux sera plus élevé; par conséquent, si l'on injecte à différents débits la même masse de soluté, la hauteur du pic variera et passera par un maximum pour un débit égal au débit optimum, correspondant à HETP minimum.

L'aire d'une courbe de Gauss est proportionnelle au produit de sa hauteur par sa déviation standard:

$$A = \sqrt{2\pi}\cdot h_{\max}\,\sigma$$

Mais, pour calculer l'aire du pic obtenu sur le chromatogramme, il faut utiliser la déviation standard exprimée en temps et non en volume, le chromatogramme étant un enregistrement tension-temps. Cette déviation standard est donnée par:

$$\sigma(t) = \frac{\sigma(V)}{D} = \frac{V_R^\circ}{D\sqrt{N}} \tag{7}$$

L'aire du pic (cm²) est donc:

$$A = \lambda_1\lambda_2\lambda_3 C_{\max}\,\sigma(t) = \lambda_1\lambda_2\lambda_3 \frac{m\sqrt{N}}{\sqrt{2\pi}\cdot V_R^\circ}\cdot\frac{V_R^\circ}{D\sqrt{N}} = \lambda_1\lambda_2\lambda_3\frac{m}{D\sqrt{2\pi}} \tag{8}$$

où λ_1 = le coefficient de proportionnalité entre l'aire d'une courbe de Gauss et le produit de sa hauteur par sa déviation standard. Ce coefficient vaut $\sqrt{2\pi}$.

λ_2 = le coefficient de proportionnalité entre la déviation de l'enregistreur et la concentration de soluté dans le gaz traversant la cellule de détection (cm/g/cm³ ou cm⁴/g).

λ_3 = le coefficient de proportionnalité entre le déplacement du papier et le temps, c'est-à-dire sa vitesse de déroulement (cm/sec). λ_3 est égal à l'inverse du coefficient C_2 de l'équation de DIMBAT *et al.*, $\lambda_3 C_2 = 1$.

L'aire du pic varie donc proportionnellement à l'inverse du débit, au moins dans la limite de validité de la théorie des plateaux. Il est normal, dans ces conditions que, suivant qu'ils aient utilisé la hauteur ou l'aire du pic, et suivant le domaine de débit employé, différents auteurs aient abouti à des conclusions contradictoires.

La conséquence directe de l'éqn. (8) est que le facteur S de DIMBAT, STROSS ET PORTER[1] se trouve bien avoir des bases théoriques solides. En effet, en remplaçant l'aire A par sa valeur ci-dessus, on obtient:

$$S = \frac{AD\ C_1C_2}{m} = \frac{1}{\sqrt{2\pi}} \lambda_1\lambda_2\lambda_3\ C_1C_2 = \lambda_2\ C_1 \qquad (9)$$

Le produit $\lambda_2 C_1$ représente le rapport entre la tension obtenue aux bornes de la diagonale de mesure du pont de Wheatstone contenant le catharomètre et la concentration du soluté dans la phase gazeuse qui traverse la cellule de détection. Autrement dit S serait la tension obtenue, si cela était possible, lorsque le gaz traversant la cellule de mesure contiendrait une concentration unité du soluté. Dans ces conditions, il paraît tout à fait naturel, comme l'a proposé YOUNG[6], de caractériser un détecteur par le rapport:

$$Q_0 = \frac{2R_n}{S}$$

qui se trouve être égal à la concentration de soluté dans le gaz vecteur correspondant à un signal égal à deux fois le bruit de fond R_n. Ce rapport est, en effet, la concentration minimale décelable de soluté dans le gaz vecteur, et détermine la hauteur minimum que doit avoir un pic pour pouvoir être décelé.

L'avantage du paramètre de YOUNG est qu'il est absolument indépendant de la colonne utilisée et qu'il caractérise le détecteur en utilisant le principe même de la mesure effectuée par ce détecteur: une mesure de concentration. La théorie ci-dessus ne préjuge en effet aucunement du mécanisme de fonctionnement du détecteur mais suppose seulement qu'il répond aux variations de concentration. Le paramètre de YOUNG permet donc également de comparer entre eux, sans difficulté, les détecteurs répondant à la concentration du soluté dans le gaz vecteur tels le catharomètre, le macro-détecteur de Lovelock, le détecteur à section de capture, la balance à densité gazeuse; par contre, il ne saurait être appliqué aux détecteurs qui répondent à la vitesse d'entrée du soluté dans le gaz vecteur, comme le détecteur à ionisation de flamme et le micro-détecteur de Lovelock.

L'utilisation pratique de la concentration minimale décelable est très facile, puisque l'on peut aisément, à l'aide de la formule (6) ci-dessus, calculer dans des conditions opératoires données, connaissant le volume de rétention d'un soluté et l'efficacité de la colonne, la concentration correspondant à la hauteur maximum du pic obtenu en injectant une masse m du soluté.

On peut en déduire inversement, connaissant la concentration minimale décelable, quelle est, dans ces conditions, la quantité minimale du composé étudié que l'on pourra détecter, en supposant que l'injection d'une telle quantité soit possible sans modifier l'efficacité de la colonne et sans que des phénomènes d'adsorption viennent modifier la valeur du volume de retention et altérer la forme du pic. La formule (6) montre que l'*on peut améliorer la sensibilité de la détection en diminuant le volume de rétention et en augmentant l'efficacité de la colonne.* Ainsi, la sensibilité sera maximale en fonctionnant au débit correspondant à la plus grande efficacité de la colonne; on aura également intérêt à utiliser des colonnes aussi courtes et peu chargées en phase que possible.

RÉSULTATS EXPÉRIMENTAUX

L'application faite aux catharomètres suppose que, pendant l'élution d'un pic, l'équilibre thermique est réalisé à chaque instant et que la différence de potentiel aux extrémités de la diagonale de mesure est toujours proportionnelle à la concentration de soluté dans le gaz vecteur. Cette hypothèse n'est qu'approchée puisque, d'après BOHEMEN ET PURNELL[7], le facteur de réponse du catharomètre doit diminuer lorsque le débit augmente, en raison de l'augmentation des pertes de chaleur dues à la différence de capacité calorifique entre le gaz vecteur pur et le gaz vecteur contenant les vapeurs du soluté.

Les expériences ont été faites en utilisant l'hydrogène comme gaz vecteur sur cinq colonnes dont on trouvera les caractéristiques dans le Tableau I. Elles ont consisté à

TABLEAU I

Colonne No.	*Support*	*Phase*	*Taux (%)*	*L (m)*	*Soluté*	*Quantité (µl)*	H_{min} *(cm)*	*Temp. (°C)*
1	Chromosorb P	Carbowax 1500 (3 g)	20	2	Méthanol	0.2	0.075	70
1	Chromosorb P	Carbowax 1500 (3 g)	20	2	Méthanol	0.2	0.125	100
1	Chromosorb P	Carbowax 1500 (3 g)	20	2	Méthanol	0.2	0.150	130
3	Téflon	Carbowax 1500 (6 g)	20	2.4	Méthanol	0.2	0.24	71
4	Chromosorb P	Diméthylsulfolane (6.5 g)	10	10	*n*-Hexane	0.4	0.075	45
5	Chromosorb P	Diméthylsulfolane (0.7 g)	5	2	*n*-Hexane	0.4	0.08	45
6	Chromosorb P	Squalane (3.2 g)	5	10	*n*-Hexane	0.4	0.11	50

mesurer dans chaque cas les variations de l'efficacité, de la hauteur et de l'aire du pic en fonction du débit, en injectant toujours la même quantité de soluté. L'appareil utilisé est un Perkin-Elmer 116 E muni d'un catharomètre à thermistances, les injections sont faites avec une seringue Hamilton de 1 µl.

La Fig. 1 montre le résultat obtenu avec la première colonne à 70°. On voit que l'aire du pic décroît régulièrement quand le débit augmente, tandis que sa hauteur passe par un maximum atteint pour le débit optimum. Le même résultat est obtenu avec les autres colonnes. Les aires sont exprimées en cm^2. Sur les chromatogrammes 1 cm en ordonnées correspond à 0.20 mV, 1 cm en abscisses à 36 sec, 1 cm^2 vaut donc 7.2 mV·sec.

La Fig. 2 montre, en coordonnées logarithmiques, les courbes de variation de l'aire du pic avec le débit. On voit que l'aire est bien inversement proportionnelle au débit. On remarque également qu'on obtient la même courbe quelle que soit la colonne si l'on a utilisé le même soluté à la même température. L'aire du pic caractérise donc bien le détecteur (et le soluté) indépendamment de la colonne.

La Fig. 3 montre, en coordonnées logarithmiques, les variations de la hauteur du pic en fonction du nombre de plateaux de la colonne. On voit que la hauteur du pic est bien proportionnelle à la racine carrée de ce nombre. On remarque également que la

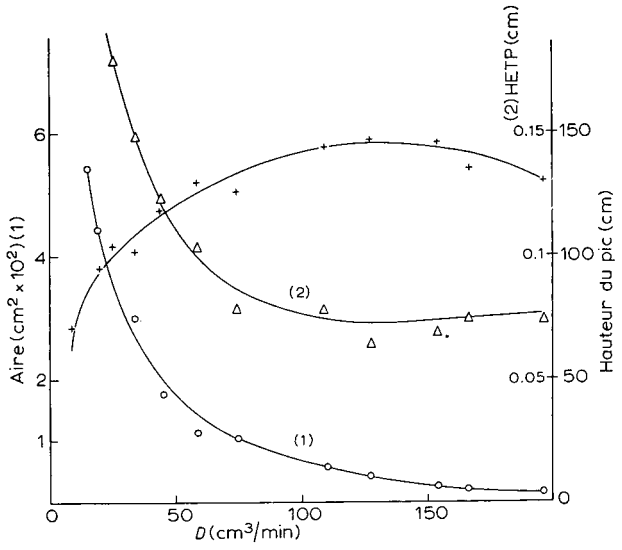


Fig. 1. Variations avec le débit, de l'efficacité (2) de la colonne No. 1 (20% Carbowax 1500 sur poudre de brique C22, à 70°), ainsi que de l'aire (1) et de la hauteur du pic obtenu après injection d'un échantillon de 0.2 mg de méthanol.

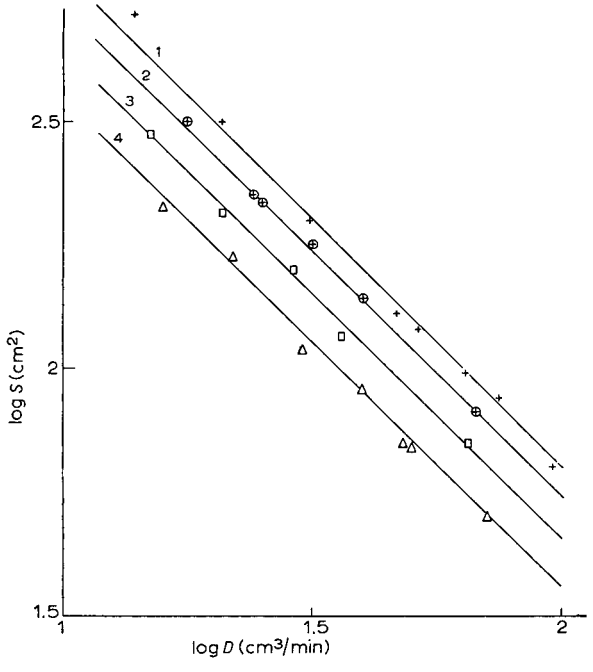


Fig. 2. Variations avec le débit de l'aire du pic obtenu sur différentes colonnes. (1) Colonne No. 1 à 70° et colonne No. 3 à 71° (0.2 μl méthanol). (2) Colonne No. 1 à 104°. (3) Colonne No. 1 à 130°. (4) Colonnes Nos. 4 et 5 à 45° (0.4 μl *n*-hexane).

hauteur du pic est, à même nombre de plateaux, 10 fois plus grande avec la colonne No. 5 qu'avec la colonne No. 6, ce qui est bien dans le rapport inverse des volumes de rétention.

Les débits maxima atteints ne dépassent pas 150 cm³/min, aussi, vu la grande différence de conductivité thermique entre l'hydrogène utilisé comme gaz vecteur et les vapeurs organiques, il est normal que l'effet signalé par BOHEMEN ET PURNELL[7] ne se manifeste pas; il est en effet beaucoup plus faible ici que lorsque l'on utilise l'azote comme l'ont fait ces auteurs.

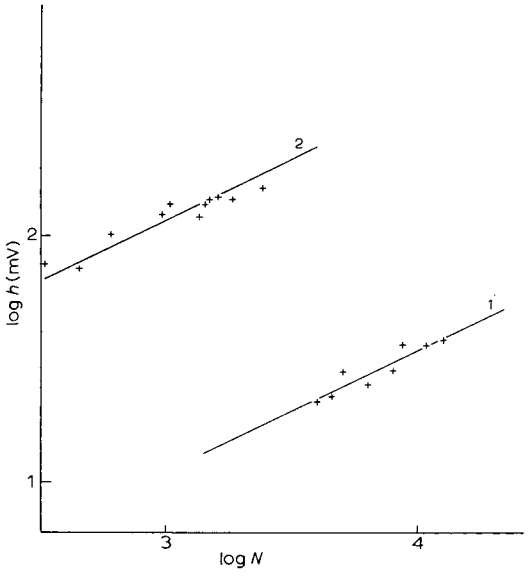


Fig. 3. Variations de la hauteur du pic obtenu avec le nombre de plateaux théoriques de la colonne. (1) Colonne No. 4. (2) Colonne No. 5.

Le Tableau II donne les résultats obtenus pour la mesure des paramètres S de DIMBAT *et al.* et Q_0 de YOUNG. On voit que Q_0 croît lentement avec la température; la variation semble à la limite des erreurs d'expériences. Si l'on exprime Q_0 en mole/cm³, la limite de détection est très voisine pour les deux solutés étudiés.

TABLEAU II

Colonne No.	*Temp. (°C)*	*Soluté*	*Quantité (µl)*	*AD* (mV·cm³)*	*$S = AD/m$ (mV·cm³/mg)*	*R_n (10^{-3}mV)*	*$Q_0 = 2R_n/S$ (10^{-6}mg/cm³)*
1	70	Méthanol**	0.2	756	4770	1	0.42
2	70	Méthanol**	0.2	756	4770	1	0.42
1	100	Méthanol**	0.2	655	4130	1	0.48
1	130	Méthanol**	0.2	540	3400	1	0.59
4	45	*n*-Hexane***	0.4	432	1640	1	1.22
5	45	*n*-Hexane***	0.4	432	1640	1	1.22
6	50	*n*-Hexane***	0.4	454	1720	1	1.16

* D'après la Fig. 2, après correction pour transformer les aires (cm²) en aires (mV·sec).
** Densité 0.793 à 20°.
*** Densité 0.660 à 20°.

CONCLUSION

Un grand nombre d'auteurs utilisent les aires des pics en analyse quantitative. D'autres préfèrent les hauteurs des pics. Les calculs ci-dessus et nos expériences montrent que, théoriquement au moins, l'aire est plus sensible aux fluctuations de débit que la hauteur du pic, lorsque la colonne fonctionne à son maximum d'efficacité. Mais le problème est plus complexe car les fluctuations de débit ne sont pas les seules à intervenir; les fluctuations de température et les variations de la manière d'injecter un même échantillon jouent un rôle. Ces dernières peuvent être particulièrement importantes lorsqu'on utilise la méthode classique de la seringue, surtout lorsqu'opérant à fort débit, on doit injecter contre la forte pression régnant en tête de colonne. Or, la hauteur du pic est beaucoup plus sensible aux fluctuations de la forme de la fonction injection que l'aire du pic. De même, la hauteur du pic étant reliée au volume de rétention est beaucoup plus sensible aux fluctuations de température de la colonne que l'aire du pic qui ne varie avec la température que parce que le facteur de réponse du catharomètre en dépend, le Tableau II montre que ces variations sont relativement faibles.

Il semble par conséquent que l'utilisation des hauteurs en analyse quantitative ne soit à recommander que lorsque les fluctuations de débit sont très importantes. A titre indicatif, avec l'appareil utilisé la déviation standard relative des aires des pics de méthanol sur la colonne No. 1 est de 1%, celle des hauteurs est de 5%. Une analyse quantitative serait donc beaucoup plus précise si les calculs étaient faits à partir des aires. Un opérateur mieux entraîné à faire des injections reproductibles, travaillant avec un appareil au débit moins stable, pourrait avoir intérêt à utiliser les hauteurs.

REMERCIEMENTS

Nous remercions Madame CATHERINE LANDAULT pour les discussions fructueuses que nous avons eues avec elle à ce sujet et Mademoiselle DENISE BOUCHEZ qui a réalisé la plupart des expériences.

RÉSUMÉ

Une étude théorique et expérimentale du facteur de réponse des catharomètres montre que l'aire du pic pour une masse de soluté donnée est proportionnelle à l'inverse du débit du gaz vecteur, et que la hauteur du pic est proportionnelle à la racine carrée du nombre de plateaux théoriques et à l'inverse du volume de rétention. Le facteur de YOUNG, qui donne la concentration minimale détectable de soluté dans le gaz vecteur, semble être le meilleur moyen de caractériser la sensibilité d'un détecteur répondant à la concentration du soluté, comme le catharomètre.

SUMMARY

A theoretical and experimental study of the response factor of catharometers shows that the peak area for a given mass of a solute is proportional to the inverse of the flow rate, and that the peak height is proportional to the square root of the plate number and to the inverse of the retention volume. The YOUNG factor, which gives the minimum concentration of a solute in the carrier gas that is detectable, appears to be

the best means of characterizing the sensitivity of a detector responding to concentration such as a catharometer.

BIBLIOGRAPHIE

[1] M. DIMBAT, P. E. PORTER ET F. H. STROSS, *Anal. Chem.*, 28 (1956) 290.
[2] A. I. M. KEULEMANS, A. KWANTES ET G. W. A. RIJNDERS, *Anal. Chim. Acta*, 16 (1957) 29.
[3] A. B. LITTLEWOOD, *Gas Chromatography*, Academic Press, New-York, 1962, Chap. 4.
[4] A. J. P. MARTIN ET R. L. M. SYNGE, *Biochem. J.*, 35 (1941) 1358.
[5] C. S. G. PHILLIPS, *Gas Chromatography*, Academic Press, New-York, 1956, p. 95.
[6] I. G. YOUNG, dans H. J. NOEBELS, R. F. WALL ET N. BRENNER (Editeurs), *Gas Chromatography*, Academic Press, New-York, 1961, p. 75.
[7] J. BOHEMEN ET J. H. PURNELL, *J. Appl. Chem.* (*London*), 8 (1958) 433.

DETECTION OF VOLUME PARTS PER MILLION OF PERMANENT GASES IN HELIUM

P. J. BOURKE, R. W. DAWSON AND W. H. DENTON

Chemical Engineering Division, A.E.R.E., Harwell, Didcot, Berks. (Great Britain)

(Received September 24th, 1963)

I. INTRODUCTION

In high temperature helium cooled graphite moderated nuclear reactors the partial pressures of chemically reactive gases (such as H_2, O_2, N_2, CO, CO_2 and CH_4) must be reduced to the order of 10^{-6} atm in order to limit chemical reaction with the moderator graphite[7]. It is therefore necessary to carry out analyses for these partial pressures in the coolant (up to total pressures of 25 atm) involving the determination of volume parts per million (v.p.m.) of the reactive gases in helium.

Gas chromatography with its advantage of rapid analysis of one sample for several gases is one of the possible analytical methods favoured. Since the object is to measure v.p.m. of other gases in samples consisting almost entirely of helium, the obvious carrier gas to use through the separation column and detector is helium itself. This eliminates the dominant helium peak which would occur on the chromatogram with any other carrier gas and which would be difficult to resolve in the column from peaks for v.p.m. amounts of other permanent gases in the sample. Separation of permanent gases at comparable low partial pressures can easily be achieved in molecular sieve and silica gel columns. The separation of gases in a sample must, however, be obtained without excessive decay of their initially low partial pressures occurring in the column due to axial diffusion or mixing and resistance to adsorption on the packing, otherwise the detection would be made more difficult. The volume of the sample, the adsorbent, the temperature and dimensions of the column and the flow rate of the carrier stream must therefore be chosen carefully to minimize this decay.

The most promising method of detection of v.p.m. of permanent gases in helium after chromatographic separation appears to be radioactive ionization. The principle of this method, as originally used by LOVELOCK[1] to detect v.p.m. of organics in argon, is that radiation from an active source (such as ^{90}Sr) placed in an ionization cell is absorbed by argon flowing through the cell to raise some atoms to a metastable energy level of 11.6 eV. These atoms collide and ionize atoms or molecules of other gases present having ionization potentials less than 11.6 eV. Electrodes in the cell at a suitable voltage difference collect the ions formed to give an ionization current dependent on the amounts of other gases in the argon. By careful arrangement of the cell geometry the increase in ionization current produced by even low v.p.m. of other gases is made comparable with the standing current for pure argon. The high sensitivity and accuracy of this method is due to this effect and to the stability of radioactive ionization in comparison with other means of ionization (such as flames).

With a metastable energy of 11.6 eV, argon is not capable of directly ionizing permanent gases all of which have ionization potentials greater than 12 eV (see Table I). Helium, however, has a metastable energy of 19.8 eV which is higher than the ionization potentials of all permanent gases except neon and should therefore be capable of ionizing these gases. BERRY[2], SERPINET[3] and KARMEN *et al.*[4] have shown that the Lovelock type of ionization cell can detect n.t.p. milli-micro litre quantities of permanent gases introduced in small volumes into a helium carrier stream. Data are lacking, however, on the sensitivity of the detector to low v.p.m. of permanent gases in helium. This detector with a helium carrier stream is therefore an obvious choice for use in chromatographic analysis of v.p.m. of permanent gases in helium providing it has the necessary sensitivity.

TABLE I

IONIZATION POTENTIALS

Gas	*Ionization potential (eV)*
He	24.5
Ne	21.5
A	15.7
CH_4	14.5
N_2	14.5
Kr	13.9
O_2	13.5
H_2	13.5
Xe	12.1

In using the detector to indicate the partial pressure of a chromatographically separated pulse of another gas in the carrier gas, the chromatographic peak observed shows an increase in ionization current above the standing value for the carrier gas as the pulse passes through the detector. This increase is not necessarily a measure of the absolute partial pressure of the pulse but only of the difference between this partial pressure and the partial pressure of any of this gas remaining in the carrier gas when noting the standing current. The observed value of the partial pressure of the pulse is in error on the small side by this latter amount. If this error is to be comparatively small the carrier gas must be so pure that the partial pressures in it of all gases, for which analysis is being made, are small compared with the values being analysed.

This paper describes an experimental programme to determine the accuracy of the Lovelock radioactive ionization cell for the detection of v.p.m. of permanent gases in a chromatographic carrier stream of purified helium. A purifier was built to reduce as low as possible the concentration of all other gases in a helium stream from commercial high pressure cylinders. Measurements were made of the purity of the helium stream and of the detector stability and sensitivity to various gases added to the purified helium stream.

2. EXPERIMENTAL

Commercial helium from high pressure cylinders usually contains up to 10 v.p.m. of various permanent gases and considerably greater amounts of water vapour. Before

using this helium as a carrier stream for chromatographic analysis for v.p.m. of permanent gases it is therefore necessary to purify it. The most powerful means of helium purification is a liquid helium-cooled (4°K) trap to freeze out all other gases and vapours to very low levels. This, however, is inconvenient for routine use. Other methods of purification are reviewed by ANTILL *et al.*[5]. BERRY[2] has combined both chemical and adsorption trapping to produce a purifier which was thought to reduce most impurities to levels comparable with or less than the limits of detection of the Lovelock detector. A similar purifier was accordingly built for the present work.

The experimental programme was divided into two main parts, investigation of the detector stability and sensitivity and investigation of the purity of the helium stream.

The detector stability and sensitivity depend respectively on the variations in ionization current

(i) occurring at random from time to time for the purified carrier stream and

(ii) produced by additions of other gases to the carrier stream.

The former can easily be studied by recording the detector standing current. Investigation of the latter requires a means of producing known v.p.m. ratios of other gases in helium. For this purpose a flowmeter mixing train was built with which it was possible to add flows of other gases to the helium carrier stream to give ratios down to $1:10^8$. The use of this train enabled a study to be made of the sensitivity of the detector to various gases. By reducing the amounts of gases added until a limit was reached at which the increase in ionization current became comparable with the random variation in the standing current the minimum significantly detectable ratios were determined.

To establish that any gases remaining in the purified stream were present only at levels comparable with or less than the limits of detection presented a problem as there was no direct method of measuring them. This difficulty was dealt with in two ways, both using a liquid helium cooled trap which had been proved to have a high trapping efficiency in the v.p.m. range for all permanent gases. Firstly, it was shown that passing the purified helium stream through this trap immediately before the detector produced no significant fall in ionization current below its value when not using the trap. Therefore, since the trap efficiency was high any gases which it removed could have been present only up to v.p.m. comparable with the detectable limits. Secondly, a large measured volume of the helium was passed through the trap, and the trap was then isolated and warmed to room temperature. The solidified gases were thereby evaporated back into the closed trap and their partial pressures were amplified by a known ratio to measurable values. The contents of the trap were then analysed by both chromatography and mass spectrometry. From the results of these analyses the original partial pressures of all other gases in the purified helium stream were calculated.

3. APPARATUS

The experimental equipment consisted of a metal purification train supplying purified helium to three pieces of all-glass apparatus. These were:

(i) The liquid helium trap for determination of the purity of the purified helium.

(ii) The flowmeter mixing train for calibration of the ionization detector for v.p.m. additions of other gases to the purified helium.

(iii) The gas sampling valves and separation column for chromatographic analysis of samples of gas mixtures.

The layout of these experiments is show diagrammatically in Fig. 1.

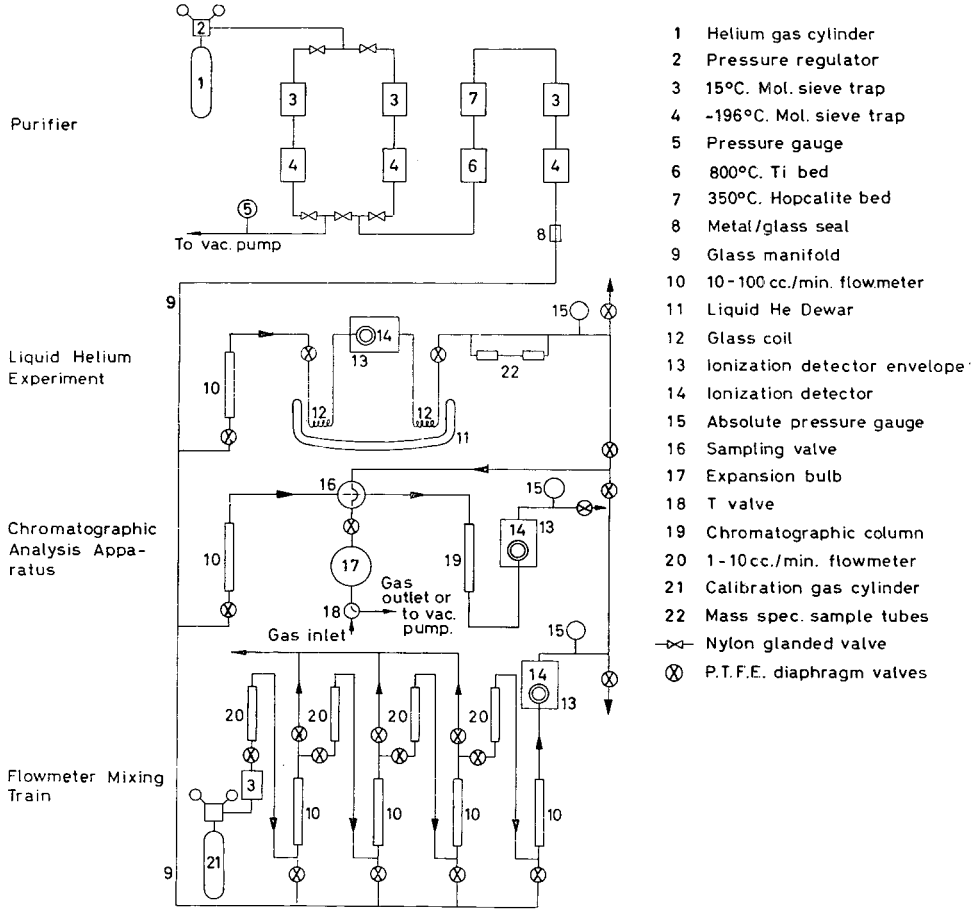


Fig. 1. Flow sheet: purifier and experimental equipment.

3.1 Purification train

The purification train, similar to that used by BERRY[2], was supplied with commercial helium from high pressure cylinders. The helium passed through the following four stages, in series (Fig. 1):

(i) Room temperature (3) and liquid nitrogen (4) cooled molecular sieve traps removed the bulk of the H_2O, CO_2 and easily condensable gases. The sieve was Linde grade 5A, $^1/_{16}$-in. pellets.

(ii) Titanium chips (6) at 800° removed N_2.

(iii) Hopcalite (7), a mixture of copper and manganese and their oxides, trapped O_2 as CuO and oxidized the H_2 to H_2O and the CO to CO_2.

(iv) Final room temperature and liquid nitrogen-cooled molecular sieve traps removed the H_2O and CO_2 formed in the previous stage.

All these six traps were 6-in. long cylinders of i.d. *ca.* 2-in. Pyrotenax heating cable was wound round the cylinders to heat them when required. The first stage molecular sieve traps, which removed the bulk of the impurities, were duplicated in parallel to allow regeneration by heating without interrupting the flow. To improve heat transfer, a $^1/_8$-in. d. coiled pipe was tried out initially for the liquid nitrogen sieve trap, but owing to its tendency to become blocked by ice plugs near the liquid nitrogen surface was discarded in favour of cylinders having $^1/_2$ -in. d. inlet pipes.

All traps and piping in the train were of stainless steel and were washed with nitric acid and acetone before assembly. Nylon glanded valves (for flow control) and compression couplings were used upstream of the second stage. From this stage onwards there were no valves or couplings and an all-welded construction was used to ensure leak tightness.

The outlet from the purification train led through a metal to glass seal to a glass manifold (9 in Fig. 1) from which the purified helium flowed to the glass apparatus. Each apparatus was attached to this manifold by glass-blowing to form an integral all-glass construction. Most of the valves used had P.T.F.E. diaphragms. The only others were those for introducing gas samples into the carrier stream before the separation column; these, being multi-way valves, were necessarily greased cocks. The flow meters used were of the glass tube rotameter type with stainless steel floats. Pressures were measured by stainless steel diaphragm absolute pressure gauges reading from 0 to 1000 mm Hg with an accuracy of $^1/_2$ mm Hg. The ionization detectors were at room temperature which in the controlled ventilation laboratory varied from 20° to 25°.

3.2 Gas sampling valve and separation columns

The apparatus for the chromatographic analysis consisted of a flowmeter to measure the carrier flow, a sample introduction valve, a separation column and an ionization detector. The sampling valve (16 in Fig. 1), was a grease cock with two pairs of diametrically opposite inlets and outlets to its body. Its barrel had two separate bores each connecting an inlet and an outlet. The carrier stream was passed through one bore and the stream to be sampled through the other. By rotating the barrel through 90°, gas from the sample stream was first trapped in its bore and then injected as a slug into the momentarily interrupted carrier flow. The column (19 in Fig. 1) was a 3 mm bore 35 cm long glass tube. It was packed with either 50–60 mesh silica gel or a molecular sieve depending on the gases to be separated. It was wrapped with heating tape which could warm it up to 200° for regeneration and operation above room temperature.

3.3 Liquid helium trap

The apparatus for the liquid helium experiment (Fig. 1) consisted of a flow-meter, a 1 mm bore 25 cm long coiled glass tube, an ionization detector and another similar coil. These coils were positioned in a liquid helium dewar which was itself surrounded by a liquid nitrogen dewar, and when cooled to the normal boiling point of liquid helium (4°K) acted as traps to freeze out other gases from the helium passing through them. The detector was placed outside the dewar with a sufficient length of tubing between

it and the liquid helium to ensure that the helium gas arriving at it had warmed from 4°K to room temperature. The second coil was used as a safeguard against back diffusion to the ionization detector from the outlet during filling with liquid helium when the contraction on cooling the gas in the coils temporarily stopped the flow. It was probably unnecessary because during filling the outlet valve was closed and the whole unit was leak tight to high vacuum standards. The outlet tube from the second coil had a parallel loop of mass spectrometry breaker seal sample tubes. When samples were required these tubes could be sealed off and removed. Further downstream the outlet flow passed through the chromatographic sampling valve (16 in Fig. 1) and finally to the atmosphere through a P.T.F.E. diaphragm valve.

In running this experiment the gas pressure and flow rate were controlled by adjustment of the P.T.F.E. valves at the beginning and end of the apparatus. The pressure was usually held at a few mm Hg above atmospheric pressure. This increase in pressure was sufficient to liquefy some of the helium gas flowing through the coils cooled by the liquid helium at its normal boiling point and made the flow erratic. To prevent this the liquid surface in the dewar was pressurised to a few cm Hg with helium. The resulting rise of about 0.1° of the liquid above its normal boiling point was then sufficient to avoid liquefying the gas in the coils.

3.4 Flow meter mixing train

For calibration experiments the flow meter mixing train was built to provide a stream of purified helium with which constant known flows of other gases could be mixed down to a ratio of $1:10^8$. It consisted of four streams of purified helium taken from the manifold though flowmeters (10 in Fig. 1) at rates up to 100 cc/min. Into the first of these streams, at a point before its flowmeter, a second gas from a cylinder and molecular sieve trap was metered through a smaller flowmeter (20 in Fig. 1) at rates down to 1 cc/min. (This is about the lowest gas flow accurately measurable by a rotameter.) The larger flowmeter both measured and mixed the two flows to give dilutions down to 1:100. From this mixture 1 cc/min was metered into the second purified helium stream to give dilutions down to 1:10,000 and so on. By using the four stages of the train it was possible to achieve dilutions down to $1:10^8$. This continuous flow method, in spite of the wastage of about three quarters of the purified helium to the atmosphere, was preferred to a batch mixing method of making up samples for two reasons. Firstly it provides a convenient and infinitely variable control of the dilution ratio. Secondly, because of the continuous flow, the amounts of gases adsorbed on the glass tube approach equilibrium with the gas phase, whereas in the batch mixing of large with small volumes of gas, there is always uncertainty as to how much the partial pressure of the dilute component may be altered by adsorption or desorption on the walls of the vessel.

The gas stream from the fourth mixing stage passed through an ionization detector and a P.T.F.E. diaphragm valve to the atmosphere. Using this apparatus it was possible to calibrate the ionization current of the detector against steady known v.p.m. additions of various gases to the purified helium stream.

A second outlet from the fourth stage of the mixing train led through the sample side of the chromatographic sampling valve. By rotating this valve through 90°, a sample of this stream was introduced into the carrier stream to the separation column. This enabled a direct calibration of either the chromatogram area or the peak height

to be made against samples of known v.p.m. addition. The calibration of peak height differed from the steady calibration because, as the sample passed through the column to the detector, the gas in it diffused forwards and backwards thus reducing its peak v.p.m.

A direct check that the mixing of the various gas streams in the flowmeter train was complete and that the calculated mixing ratios were accurate was considered desirable. This was done using the chromatographic analysis apparatus and the following device. A glass expansion bulb (17 in. Fig. 1) with two outlets was glass blown into the line from the sample side of the chromatographic sampling valve (16 in Fig. 1). The other outlet from the bulb led to one of the arms of a "T" valve (18 in Fig. 1) with a "half moon" bore through its barrel. The bulb was connected through one arm and the leg of the "T" valve to a vacuum pump. After evacuation the bulb was isolated under vacuum by rotating the barrel of the "T" valve clockwise to a position where it connected the leg and the other arm. A gas stream was then passed through its bore. Further clockwise rotation of the barrel isolated the bore filled with gas until it opened only to the bulb. The bore full of gas then expanded into the bulb and sample bore of the sampling valve (16 in Fig. 1). The volume ratio of the expansion was about 1:4,000. Hence if the gas stream had been passed at one atmosphere through the "T" valve, the sample bore of the sampling valve was filled with gas at a pressure of 250×10^{-6} atm. (This pressure could be increased in units of 250×10^{-6} atm by repeating the latter part of the above procedure.) By rotating the barrel of the sampling valve a sample of the gas at this pressure was introduced into the column carrier stream. The chromatogram thus obtained was compared with that obtained by sampling 250 v.p.m. of the same gas in helium at one atmosphere, prepared by the flowmeter train, since both samples of the same volume and temperature should be at the same partial pressure, and, provided the mixing train was operating accurately, should therefore contain the same mass.

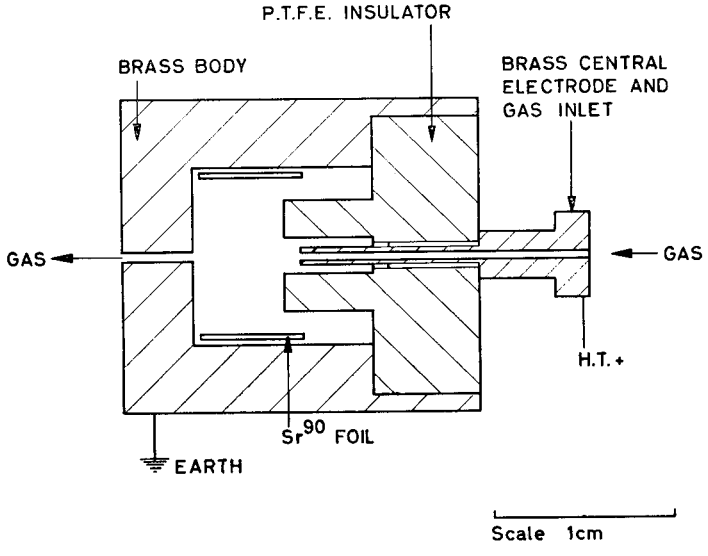


Fig. 2. Detector.

3.5 Detectors and electronics

The three ionization detectors used were commercial argon micro detectors of W. G. Pye Ltd., Cambridge. As supplied for use with small carrier flows from capillary columns each detector casing had a second inlet intended for the admission of a scavenge flow. This was not necessary in the present work with high carrier flows and the second inlets were blanked off. The detectors contained 10 mC ^{90}Sr radioactive sources. Their dimensions are shown in Fig. 2.

Because they were not leak tight to high vacuum standards they were totally enclosed in glass envelopes for the present work. The voltage applied to the detectors was obtained from a power pack which provided voltages of 750, 1000, 1250 and 1500 V. The current from the detector was amplified for recording by a Honeywell Brown 10 mV recorder. The amplifier had various ranges with which full scale deflection on the recorder could be obtained for currents in the range 1.4×10^{-9} to 4.7×10^{-7} amps. The amplifier also had several ranges of attenuation with which up to 95 % of the current could be backed off leaving only 5 % for amplification. This was useful for the analysis of the lowest v.p.m. which gave changes of current comparable with the standing value.

4. RESULTS

4.1 Commissioning experience

After the equipment described above had been built and satisfactorily pressure and vacuum tested the molecular sieve traps were regenerated to drive off adsorbed gases. Regeneration was carried out by holding the traps at 300° under vacuum for 12 h. After this period dew point measurements of a helium stream passed through the hot taps showed the H_2O vapour pressure to have fallen to 0.9 mm Hg. From Linde data[6] for H_2O adsorption on molecular sieves this indicates an H_2O weight loading of the sieve of 1 %. This weight loading at 15° has an H_2O vapour pressure in equilibrium with it of 5×10^{-5} mm Hg. Extrapolation of the data to —196° indicates a quite insignificant equilibrium vapour pressure.

The purification train was then put into service at 3–5 lb/in^2 g pressure of helium. During the commissioning period and first attempts of the analytical experiments several modifications to the pipe work layout were made to give the final form shown diagrammatically in Fig. 1. Experience gained during this period showed that when the purification train was started up after exposure to the atmosphere the outlet helium usually contained up to 1000 v.p.m. of impurities. This ratio then decreased over a period of hours to some 50–100 v.p.m. as the impurities diffused out of dead pockets in the pipe work. After this time the ratio continued to decrease but at a very much slower rate as impurities adsorbed on the pipe walls, desorbed into the helium stream. The rate of degassing the walls could be greatly increased by flaming the glass and metal pipes to red heat and heating the P.T.F.E. valves to 200°. Usually, after two to three days the standing current of the ionization detectors had fallen to a steady minimum value.

The purification train has been operated continuously with helium flows of between 100 and 500 cc/min. This experience shows that the first stage molecular sieve traps have sufficient capacity for up to a month's running without letting impurities break through. The fourth stage sieve has operated satisfactorily for up to

six months without regeneration. No trouble has been experienced with the titanium and hopcalite stages.

After the modifications and trials were complete the purification train was run without stop for three months with purified helium always flowing through all the glass ware. The experiments described below were carried out during this period.

4.2 Detector stability and sensitivity

The experimental programme began with an investigation of the effects of the variables which influenced the standing current of the detector for helium from the purification train. These variables were pressure, temperature, voltage and helium flow rate.

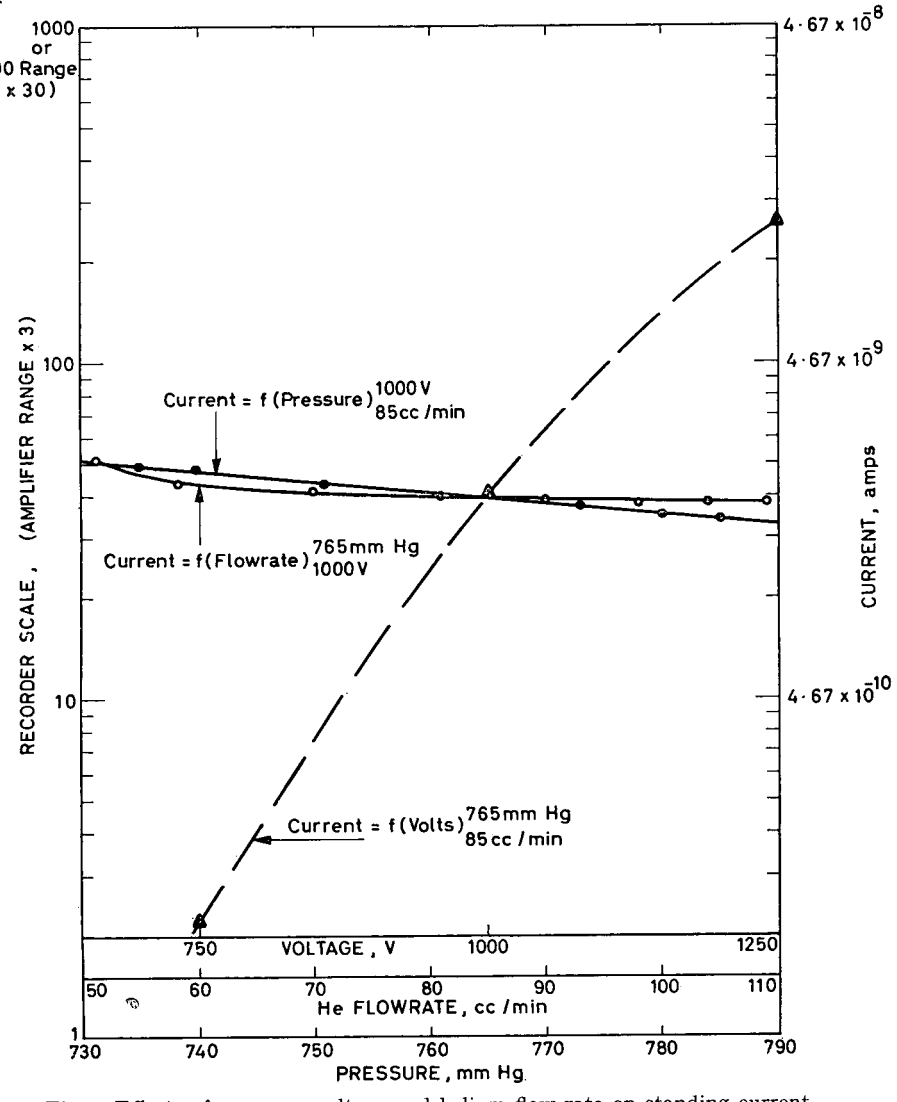


Fig. 3. Effects of pressure, voltage and helium flow rate on standing current.

The standing current was found to be approximately inversely proportional to pressure, in the range 730 to 800 mm Hg, doubling for a decrease of 90 mm Hg (Fig. 3). The effect of variation of temperature in the range 20 to 25° was such that provided the pressure was also varied to keep the helium density constant the standing current was unchanged. Increase in voltages up to 1250 V applied to the detector gave steady increases in standing current (Fig. 3). Higher voltages and even 1250 V at pressures below 750 mm Hg appeared to produce spark discharges with momentarily very high currents. Increasing the helium flow rate up to 60 cc/min slightly decreased the standing current, but from 60 cc/min up to at least 110 cc/min the standing current was independent of flow rate (Fig. 3).

From these results it was decided to select standard conditions for all further measurements. Pressure and temperature of 765 mm Hg and 23° were chosen as conveniently practical values. A voltage of 1000 V was chosen, as being the highest voltage which would give maximum sensitivity without causing spark discharge. A flow rate of 85 cc/min was chosen because of the insensitivity of the standing current to flow rates between 60 and 110 cc/min.

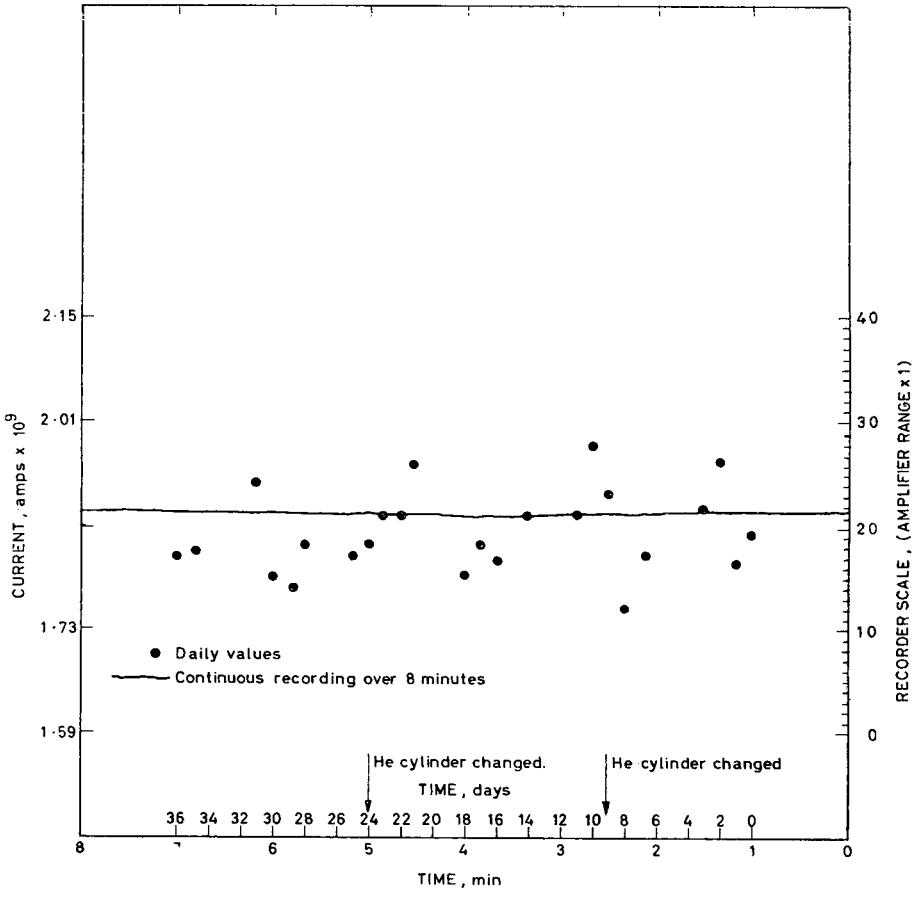


Fig. 4. Stability of standing current at standard conditions.

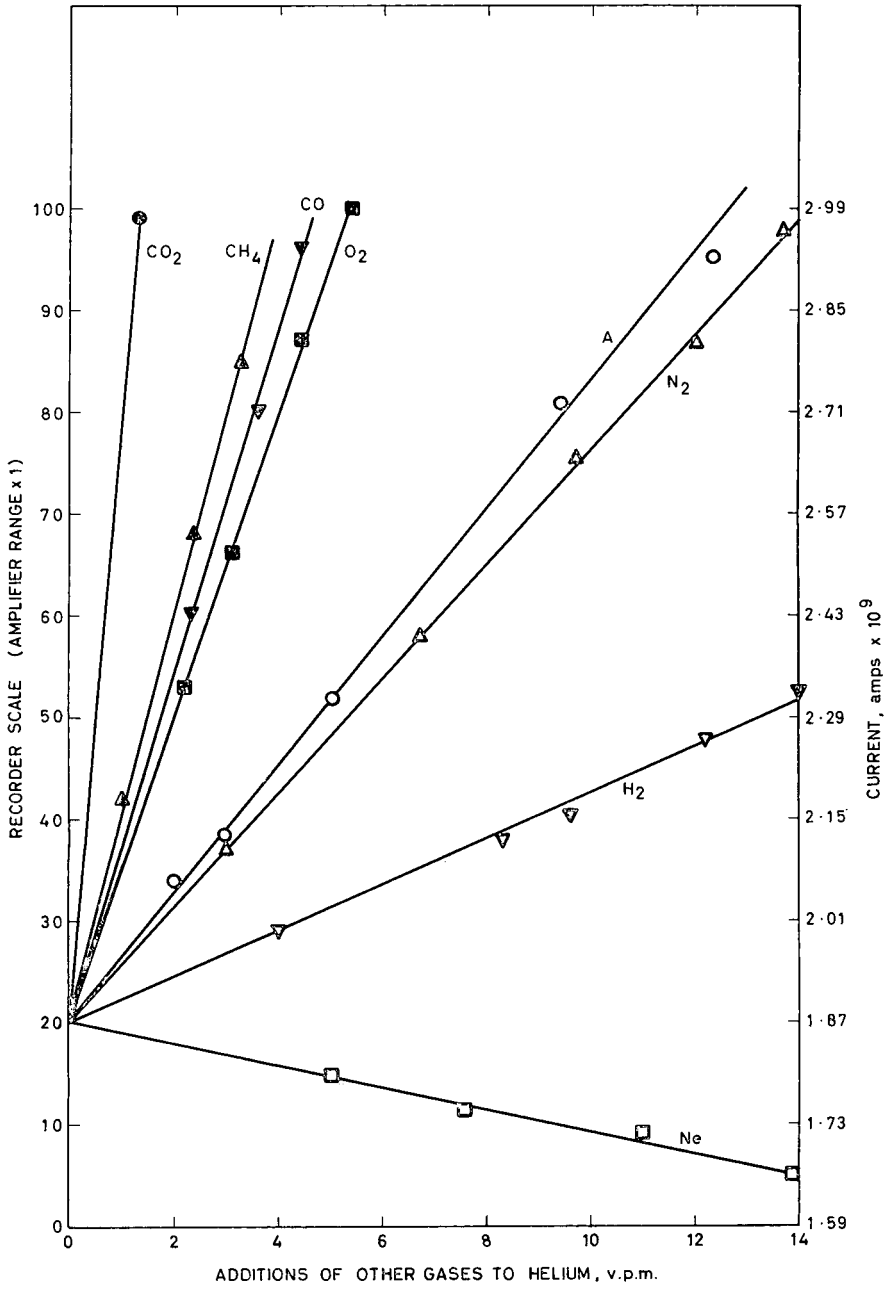


Fig. 5. Calibrations of detector.

The stability of the standing current was investigated next under standard conditions. The maximum amplification available from the electronics was accordingly used with appropriate backing off so that one scale division on the recorder chart

was equivalent to a change in current of 1.4×10^{-11} amps. An example of the short term stability is shown by the continuous line (Fig. 4) drawn on the chart over an interval of 8 min. The random variations on this line are obviously sufficiently small to make a change of one scale division significant. The long term stability is shown by the point values (Fig. 4) plotted daily over the 36 days during which these experiments were carried out. The variation of the points may have been due either to variations in the purity of the helium from the purification train or to inaccuracies in setting the standard conditions.

Calibrations of the detector sensitivity to various gases were made using the flow meter train to mix constant known v.p.m. additions of other gases with the purified helium stream. Increases in ionization current are plotted against v.p.m. additions (Fig. 5). The results show that the sensitivity is linear over the ranges plotted and varies with different gases, decreasing in the order CO_2, CO, O_2, A, N_2, H_2, Ne. Ne was exceptional in that it reduced the ionization current below its standing value. This might be explained by the fact that Ne is the only gas having an ionization potential (21 eV) higher than the metastable energy of helium (19.8 eV).

A check on the accuracy of the flowmeter train was made using the equipment for the chromatographic analysis apparatus. The helium stream with v.p.m. additions of air from the flowmeter train was passed through the sample bore of the chromatographic sampling valve (16 in Fig. 1). Samples of this stream were introduced into the carrier stream to the column. The chromatogram obtained showed peaks of O_2 and N_2. The areas under the peaks were determined by cutting the peaks out of the chart and weighing. The areas obtained are plotted (in arbitrary units) against O_2 and N_2 partial pressures in the sample, in Fig. 6. The "T" valve (18 in Fig. 1) was then used to fill the sample bore of the sampling valve (16 in Fig. 1) with air at known low pressures as described in 3.4. Samples of this low pressure air were introduced into the column carrier stream. The areas under the O_2 and N_2 peaks of the chromatograms obtained were again determined by weighing and are plotted in Fig. 6.

Comparison of the two sets of results is made on areas which are proportional to the masses of O_2 and N_2 in the samples rather than peak heights. This was necessary because the samples from the flowmeter train were in helium at atmospheric pressure, whereas the samples from the "T" valve were under vacuum. Slightly different peak widths were obtained in the two sets of results, and comparison of the areas for the two methods of making up the samples showed that the differences had standard deviations of 5 % for O_2 and 6 % for N_2.

These standard deviations are about the expected values, considering that each of the flowmeters in the train had a standard deviation of 2 %. In calculating the v.p.m. additions made by the train, eight flows were multiplied and divided together. Elementary statistics show that the result should have a standard deviation of $\sqrt{8} \times 2\%$ or 5.7 %.

In view of this, the standard deviation of the calibrations on Fig. 6 cannot be considered better than about 6 % even though the actual points plotted show a smaller deviation. This is due to the way the flowmeter train was operated to give different v.p.m. additions by varying only one or two flowmeters while the others remained constant, instead of randomising all values.

The stability of the detector standing current may now be assessed in terms of v.p.m. additions of other gases to the purified helium. From Fig. 4 it is evident that

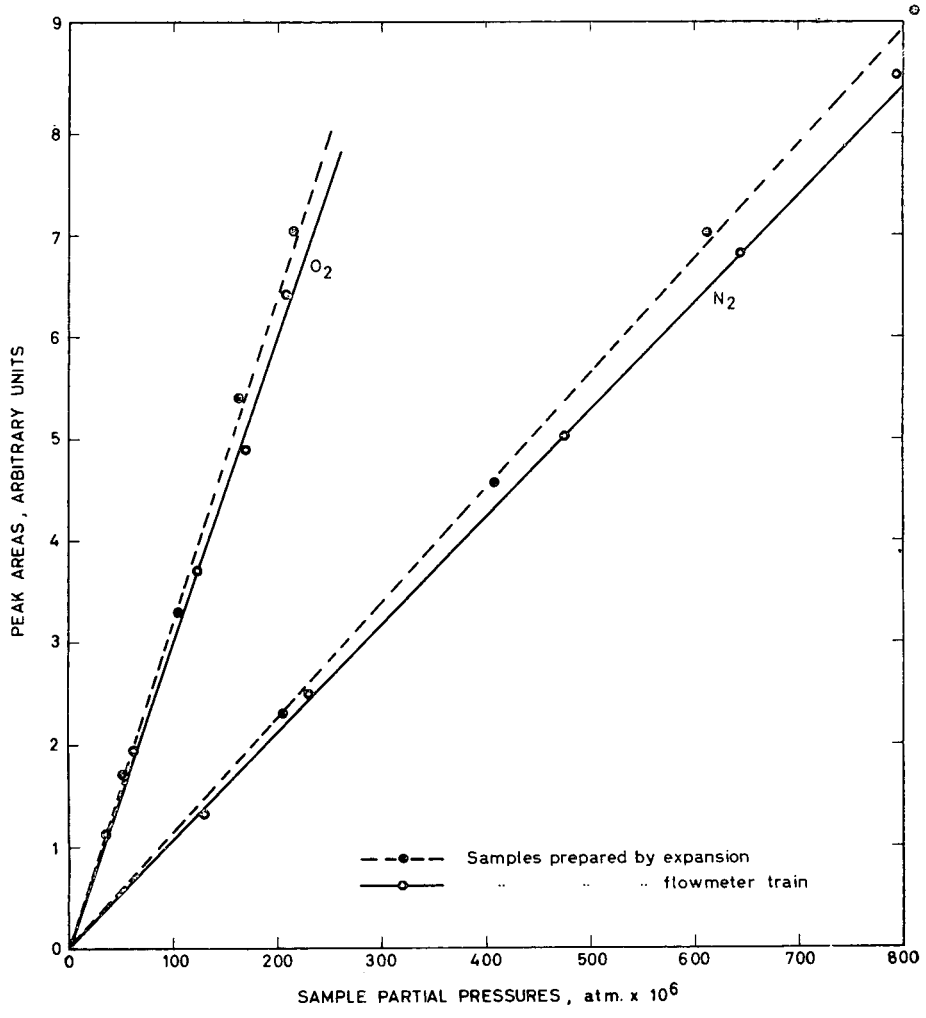


Fig. 6. Comparison of chromatograms of samples prepared by expansion and by flowmeter train.

the short term stability is such that an abrupt change in ionization current of 1.4×10^{-11} amps (one scale division) significantly indicates a change in the v.p.m. of other gases in helium stream. The v.p.m. of various gases equivalent to 1.4×10^{-11} amps change (taken from Fig. 5) are:

CO_2	CH_4	CO	O_2	A	N_2	H_2	Ne
0.02	0.05	0.06	0.07	0.15	0.2	0.5	1.0

These are the minimum significantly detectable v.p.m. changes of other gases in the purified helium.

The results given in Figs. 4 and 5 were obtained from the detector used for the liquid helium experiment (4.3) using an outlet from the flow meter train not shown in Fig. 1.

4.3 Helium purity

Before carrying out the liquid helium experiment to investigate the purity of the helium gas from the purifier it was necessary to establish that the trapping efficiency and capacity of the liquid helium cooled coils (12 in Fig. 1) were adequate to reduce to sufficiently low levels all other gases which might remain in the purified helium. The saturated vapour pressures of permanent gases were extrapolated to 4°K, using the Clausius-Clapeyron equation. This showed that, if equilibrium was attained in the coils, H_2 and Ne ratios in the helium at one atmosphere would be reduced to about 10^{-5} v.p.m. and 10^{-9} v.p.m., respectively, and ratios of all other gases would be quite negligible. Equilibrium may not, however, be attained because nucleiation of impurities in the gas may result in solid aerosol particles blowing through the coils and re-evaporating on warming downstream. The trapping efficiency and capacity of the coils had therefore to be determined experimentally. This was done by filling the dewar (11 in Fig. 1) with liquid helium to cover the coils. After temperatures and the purified helium gas flow had steadied, the standing current of the ionization detector was noted. Then, using the flowmeter mixing train, additions up to 50 v.p.m. of various gases were made to the purified helium stream before the coils for periods up to one hour. For no gas, at any v.p.m., was there any detectable change in the standing current. It was therefore concluded that the liquid helium cooled coils had adequate efficiency to remove all other gases from the helium to at least as low as the minimum detectable changes (4.2). The capacity of the coils was also shown to be more than sufficient to hold all the gases which were removed from the purified helium during an extended run.

After the trapping performance of the coils had been checked, the first part of the liquid helium experiment was begun by recording (Fig. 7) the standing current of the ionization detector for the purified helium passing through the coils at room tempera-

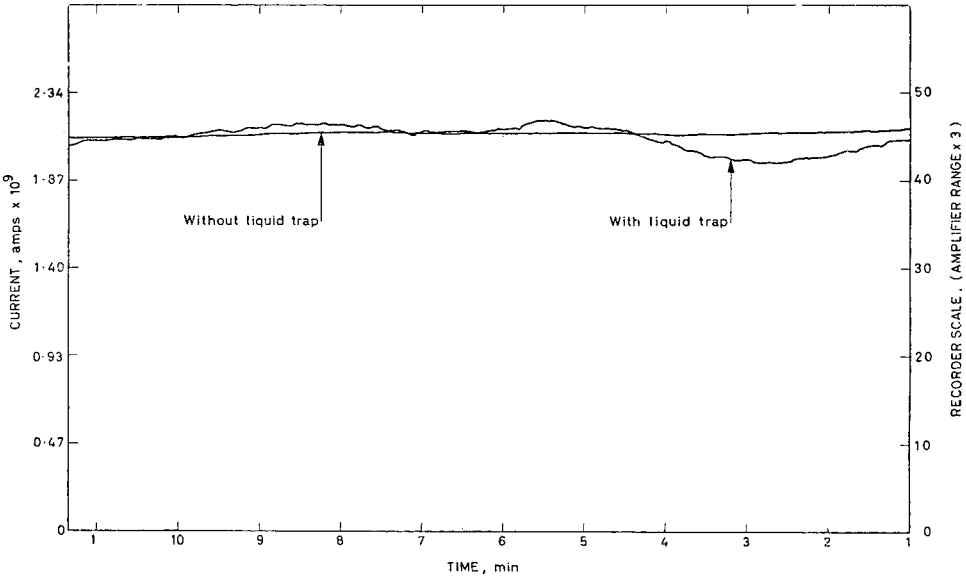


Fig. 7. Standing current with and without liquid helium trap.

ture. The coils were then cooled to 4°K and the standing current was again recorded on the same chart (Fig. 7) at the same pressure, temperature and flow rate. The line on Fig. 7 for the coils at room temperature shows the usual stability of the standing current. The line for the coils at 4°K has considerably greater fluctuations. These seemed to be caused by pressure and flowrate variations of the gas stream induced by even the smallest draughts in the laboratory near the gas stream outlet. They occurred when the coils were at 4°K and only slightly above ambient pressure (to prevent liquefaction of the gas by the liquid helium). It was probable that pressure and flowrate surges may have been the result of pressure variations downstream causing slight displacement through the large temperature gradients along the glass tubes dipping into the liquid helium of the large mass of gas near saturation and at about 1000 times its normal density held up in the coils.

Treating the differences between the two lines on Fig. 7 statistically, the liquid helium standing current has an average value 1.9×10^{-11} amps less than the room temperature value and the standard deviation of the difference between the lines is 6.1×10^{-11} amps. Hence the difference between the lines is not significant and may have been due to the fluctuations mentioned above. Taking twice the standard deviation as significant and the calibrations of the detector (Fig. 5) for various gases, it is concluded that the liquid helium cooled coils cannot have removed from the purified helium more than any of the following v.p.m. of other gases separately:

Ne	H_2	N_2	A	O_2	CO	CH_4	CO_2
7.6	3.6	1.4	1.3	0.5	0.5	0.4	0.1

To specify upper limits to the v.p.m. of other gases in the helium before the 4°K trap the minimum detectable changes (see 4.2) must be added to these values, because the trapping efficiency of the coils were not proved better than these changes. This gives the v.p.m. upper limits as:

Ne	H_2	N_2	A	O_2	CO	CH_4	CO_2
8.6	4.1	1.6	1.5	0.6	0.6	0.4	0.1

The second part of the liquid helium experiment was an attempt to determine the actual values of the v.p.m. of the individual gases below these limits. In it the liquid helium run was extended up to five hours. After this time the two diaphragm valves shown on Fig. 1 before and after the coils were closed to isolate their contents of helium and trapped gases. The downstream section of tubing was next evacuated and closed under vacuum by the diaphragm valve immediately after the chromatographic sampling valve (16 in Fig. 1). The coils were then opened to this evacuated section, warmed to room temperatures and left for an hour to allow the trapped gases to evaporate and mix uniformly with the helium. The final helium pressure in this closed volume was about $^1/_{10}$ atm. The volume of helium passed through the coils during the run was about 150 times the closed volume. The final pressures of the trapped gases were, therefore, about 150 times the reductions in these pressures achieved by the coils during the run. To be more accurate the final impurity pressures, in addition to the 150 times amplified reductions, also included $^1/_{10}$ of the impurity pressure remaining in the helium after passing through the coils. These corrections although unknown cannot however have been more than $^1/_{10}$ of the minimum detectable changes and are negligible in comparison with the 150 fold amplification.

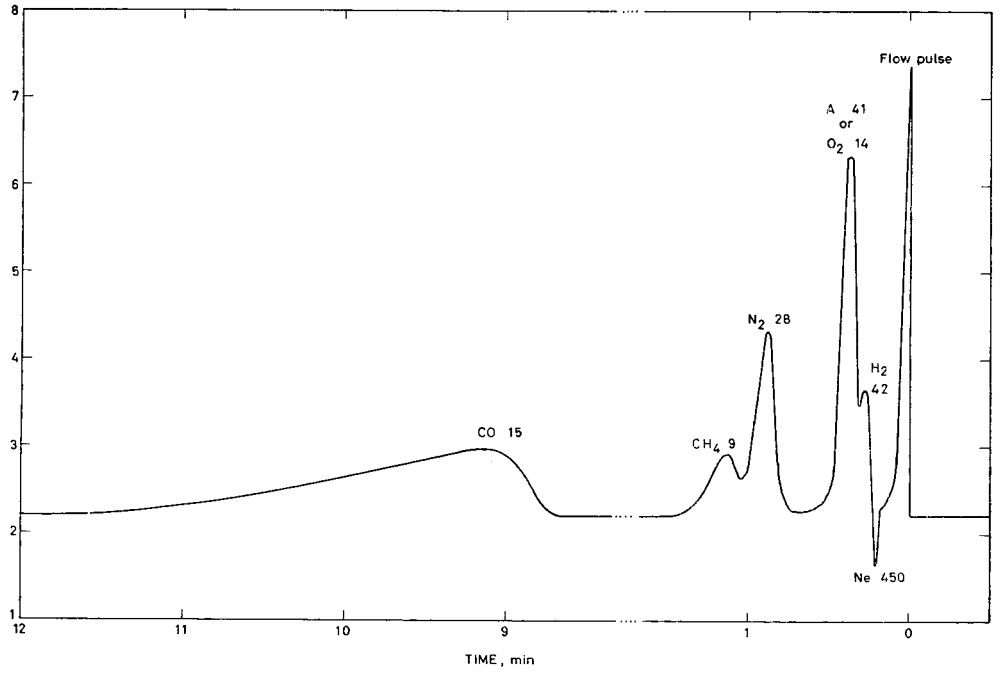


Fig. 8. Chromatogram of liquid helium trapped impurities showing their partial pressures (atm. $\times 10^{-6}$) in sample.

Mass spectrometric and gas chromatographic analyses were made of the final mixture of helium and amplified impurities. Samples for the former were taken in the breaker seal tubes (22 in Fig. 1). A sample for the latter was injected by the chromatographic sampling valve into the carrier stream to the room temperature molecular sieve column (19 in Fig. 1). The chromatogram obtained is reproduced in Fig. 8. The partial pressures in p.p.m. of one atmosphere of the various impurities in the sample were determined by calibration, using the flow meter train and are marked on Fig. 8. Peaks were observed for Ne, H_2, A or O_2, N_2, CH_4 and CO. CO_2 would not have passed through the molecular sieve at room temperature.

From the results of the two analyses the v.p.m. of the various gases removed from the helium passing through the 4°K trap were calculated to be:

	Ne	A	O_2	N_2 + CO	H_2	CH_4	CO_2
Mass spectrometry	4.5	0.7	<0.01	0.4	0.5	<0.01	<0.01
	Ne	A or O_2		N_2	CO	H_2	CH_4
Gas chromatography	3	0.3 or 0.1		0.2	0.1	0.3	0.005–
	Ne	A	O_2	N_2	CO	H_2	CH_4	CO_2
Averages (rounded)	4.0	0.5	<0.01	0.3	0.1	0.4	0.005	<0.01

N_2 and CO could not be discriminated in a mass spectrometer because of their similar molecular weights. A and O_2 could not be separated in the column because of their similar adsorption isotherms on room temperature molecular sieve.

Comparing the two sets of results there is reasonable agreement between them. The average value of each gas may, therefore, be taken as an estimate of its v.p.m. in the helium from the purification train before entering the 4°K coils if these coils are assumed to have trapped each gas to substantially less than this v.p.m. This assumption could not be proved (in the test mentioned at the beginning of 4.3) in the case of CO_2, CH_4, CO and O_2 because the estimates of their v.p.m. are less than the minimum detectable values and they might not have been efficiently trapped. Although this seems unlikely because no break through the coils was observed for inlet v.p.m. from the minimum detectable changes up to at least 50 v.p.m., to be accurate, the minimum detectable changes (see 4.2) must be added to the average values to specify upper limits to the concentrations of the various gases remaining in the helium from the purification train. This gives these v.p.m. as:

Ne	A	H_2	N_2	O_2	CO	CH_4	CO_2
5	0.7	0.8	0.5	<0.08	<0.07	<0.05	<0.03

The whole of this liquid helium experiment was carried out three times using different cylinders to supply the helium gas for purification. The results for N_2, H_2, O_2, CO, CH_4 and CO_2 were closely the same each time. The A ratios varied from 0.3 to 1.0 v.p.m. and the Ne from 3 to 5 v.p.m. The A would have been reduced only by the $-196°$ molecular sieve traps in the purifier and the Ne would not be changed by any of the purifier stages. These differences were therefore probably due to variations in the supply.

5. CONCLUSIONS

From the results of 4.3 it is concluded that the gas purification train described is capable, without the use of a final liquid helium trap, of providing a helium stream containing less than the following v.p.m. of other gases:

CO_2	CH_4	CO	O_2	N_2	A	H_2	Ne
0.03	0.05	0.07	0.08	0.5	0.7	0.9	5.0

The results of 4.2 show that when various gases are added to the purified helium, the minimum significantly detectable v.p.m. changes are:

CO_2	CH_4	CO	O_2	N_2	A	H_2	Ne
0.02	0.05	0.06	0.07	0.2	0.2	0.5	1.0

Detection by this technique of any of these gases in a helium chromatographic stream down to their minimum significantly detectable v.p.m. is therefore possible subject to constant negative errors of their v.p.m. remaining in the helium stream and to random fractional errors of 6% standard deviation due to the method of calibration.

SUMMARY

A technique is described for analysing volume parts per million (v.p.m.) mixtures of permanent gases in helium using the Lovelock radioactive ionization detector after chromatographic separation. The amounts of all other gases remaining in a carefully

purified helium carrier stream were proved using a liquid helium trap to be less than 1 v.p.m. (except neon, 5 v.p.m.), varying from 0.03 v.p.m. CO_2 to 0.9 v.p.m. H_2. The accuracy of the technique is specified down to the limits of significant detection, ranging from 0.02 v.p.m. CO_2 to 0.5 v.p.m. H_2.

REFERENCES

[1] J. E. LOVELOCK, *J. Chromatog.*, 1 (1958) 35.
[2] R. BERRY, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962, p. 321.
[3] J. SERPINET, *Anal. Chim. Acta*, 25 (1961) 505.
[4] A. KARMEN, L. GIUFFRIDA AND R. L. BOWMAN, *J. Chromatog.*, 9 (1962) 13.
[5] J. E. ANTILL, D. BRADLEY, R. M. FLOWERS AND N. HODGE, in *Progress in Nuclear Energy. Series IV. Technology, Engineering and Safety*, Vol. 4 (C. M. NICHOLLS, Editor), Pergamon, Oxford, 1961, p. 63.
[6] *Water Adsorption Isotherm Data Sheet* No. 5A-1, Union Carbide Inc., U.S.A.
[7] P. J. BOURKE AND W. H. DENTON, Carbon transport and coolant purity in high temperature gas cooled reactors, *Nuclear Reactor Chemistry, 2nd Conf., Gatlinburg, Tenn., U.S.A., 1961*, (TID 7622).

CHROMATOGRAPHY OF LIPIDS IN PRESENCE OF AN ANTIOXIDANT, 4-METHYL-2,6-DI-*tert.*-BUTYLPHENOL

J. J. WREN AND ANNA D. SZCZEPANOWSKA
The Lyons Laboratories, London (Great Britain)

(Received September 20th, 1963)

INTRODUCTION

The need for avoiding autoxidation in lipid research is well recognized. Numerous authors mention extraction, manipulation, and storage of lipids under inert atmospheres and addition of antioxidants such as hydroquinone[1]. Use of inert atmospheres is often tedious, and even traces of oxygen (as present in commercial, "high-purity" nitrogen) can produce enough peroxides to cause extensive degradation during storage. Antioxidants, on the other hand, are easily added and provide a useful period of stability towards oxygen if added before any autoxidation has begun. The useful antioxidants are chemically stable compounds, effective in small proportions, and readily separable from lipids.

Precautions taken against autoxidation during chromatography have mostly been limited to de-aeration of solvents and adsorbents, and use of nitrogen and carbon dioxide atmospheres (see *e.g.* refs.[2–8]). We now find that an antioxidant, 4-methyl-2,6-di-*tert.*-butylphenol (BHT), can be included at effective concentration in solvents used for lipid chromatography without affecting elution behaviour.

PROPERTIES OF THE ANTIOXIDANT

Chemical

BHT belongs to the class of "hindered phenols"[9], so called because their hydroxyl groups are nearly enveloped by *tert.*-butyl groups. Characteristic phenolic reactions are hindered[10]; strong bases and even metallic sodium detach protons very sluggishly. BHT is stable towards molecular oxygen except in basic media. There it is more stable than non-hindered phenols, but fairly readily oxidized to an unstable hydroperoxide, which tends to decompose back to BHT[11] and to products that are mostly stable and non-polar[12].

Chromatographic

BHT is very soluble in organic solvents, insoluble in water. It is not eluted from silicic acid by light petroleum or cyclohexane, but is eluted by carbon tetrachloride. Thus it behaves[8] like an aromatic hydrocarbon such as dodecylbenzene[13]. Like squalene and wax esters, it is eluted by 1 % diethyl ether in light petroleum, before long-chain fatty acid methyl and steryl esters. It moves ahead of methyl esters on Silica Gel G plates.

Spectroscopic

Hydroxyl stretching absorption[14–16] indicates remarkably little tendency to form hydrogen bonds: this explains the hydrocarbon-like chromatographic properties. Hydroxyl (3650 cm^{-1}) and other infrared absorptions[17] are useful for detecting and estimating BHT. So, too, is the ultraviolet spectrum (2 peaks near 280 mμ). Colorimetric assays have been described[18]. We find that a useful specific test is the blue colour ($\lambda_{max}^{CHCl_3}$ 635 mμ, $\varepsilon_a \sim 10$) of a BHT–tetracyanoethylene charge transfer complex.

Biochemical

In many countries BHT is an approved food additive, and it is fairly non-toxic to whole animals[19,20]. Unlike the natural antioxidants, tocopherols, it does not inhibit legume lipoxidases[21].

Stabilization of solvents

Ethers are stabilized by 0.01 % BHT[22]. To inhibit phosgene formation chloroform normally contains ethanol (0.75–2 %) which, because of its high polarity, sometimes affects elution behaviour. Ethanol can be replaced with advantage by BHT. Testing for phosgene by infrared absorption (1810 cm^{-1}) and smell, we find that BHT in concentrations as low as 0.001 % is an effective stabilizer. It would probably also stabilize other solvents such as dichloromethane.

APPLICATIONS

Column chromatography of wheat flour lipids

Some glycolipid and phospholipid fractions isolated from flour by published methods[23,24] gave ultraviolet spectra characteristic[3] of autoxidation products. Since the extracts are well protected by natural antioxidants until chromatographic separation begins, we considered adding another antioxidant in the eluent. BHT, which is not adsorbed from the eluents used (chloroform, methanol) gave satisfactory results at 0.005%, w/v, concentration. In numerous experiments we have detected no effect of BHT on gravimetric or spectrophotometric[23] elution curves.

Column chromatography of lipid pigments

When aliquots of a washed lipid extract from spinach leaves were developed with chloroform (containing ethanol) on identical silicic acid columns, in presence and absence of BHT (0.005%), there were many differences in the coloured band pattern. Thus it appears that the degradation of pigments reported by ZILL AND HARMON[25] was at least partly prevented by BHT.

Column chromatography of phosphatidylethanolamines

Hen's egg phosphatidylethanolamines are highly susceptible to autoxidation[3], which proceeds so rapidly that eluates turn brown after only a few hours' exposure to air in a fraction collector. But they remain colourless if BHT is present.

We have failed to detect any autoxidation of phosphatidylethanolamines occurring actually within silicic acid columns, but protection of eluates by BHT is valuable, as can be seen from Fig. 1. Rapid rechromatography incurred *ca.* 3 %

losses, probably accounted for by tailing. But when an eluate was exposed overnight there was an abrupt change to greater losses (*ca.* 17%) on rechromatography. BHT prevented the change.

Column chromatography of methyl esters

BHT (0.005%) does not speed or retard elution of methyl esters from silicic acid by 1% ether in light petroleum. Even without adding BHT we have failed to detect any autoxidation of methyl esters, even when these were shaken for 3 days with silicic acid and light petroleum in air, under fluorescent strip lighting. Yet 23% of the esters used (prepared from egg phosphatidylethanolamines) were tetra-, penta-, and hexaenoates. This contrasts sharply with the findings of earlier workers[2]. It might possibly be explained by the purity of our adsorbent, or by permeation of BHT through the laboratory. (BHT is quite volatile, see below).

Thin-layer chromatography of phosphatidylethanolamines

When unsaturated lipids are left on thin-layer plates for some time before development minor zones and streaks subsequently appear, owing to autoxidation. Rapid handling is usually practicable for single-development chromatograms but not for two-dimensional chromatograms. Incorporation of BHT in developing solvents gives the necessary protection. The photographs in Fig. 2 provide a striking if somewhat exaggerated illustration: both plates were left for 20 h between developments.

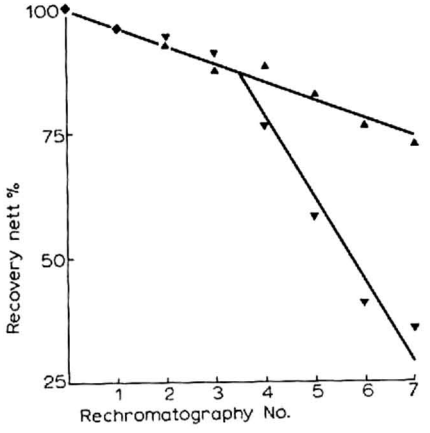


Fig. 1. Protection of phosphatidylethanolamines by BHT during silicic acid chromatography. The following series of experiments was conducted simultaneously on two identical lipid samples, with BHT (0.005%) present in eluents for one sample (▲) but not for the other (▼). Washed, total hen's egg lipid (575 mg) was applied to a column of silicic acid (15 g, diam. 33 mm) prepared in chloroform. After washing with 10% (v/v) methanol in chloroform (100 ml), phosphatidylethanolamines were eluted with 15% methanol (200 ml). The eluate was immediately evaporated ($< 40°$), and the residue dissolved in chloroform and made up to 25 ml. 1 ml solution was taken for measurement of ester absorption (1745 cm^{-1}). The remaining 24 ml was rechromatographed on a new column (5 g, diam. 23 mm), washed with 5% methanol (40 ml), and eluted with 15% methanol (50 ml). This eluate was evaporated and made up to 10 ml chloroform solution, 1 ml of which was taken for absorption measurement. The remaining 9 ml was rechromatographed on a new column (5 g), and so on. The first four measurements were made in 1 day. But then, in the third rechromatography, the eluate was collected and stood overnight. Four more measurements were made during the second day. Nett recovery includes allowance for the 1 ml samples removed.

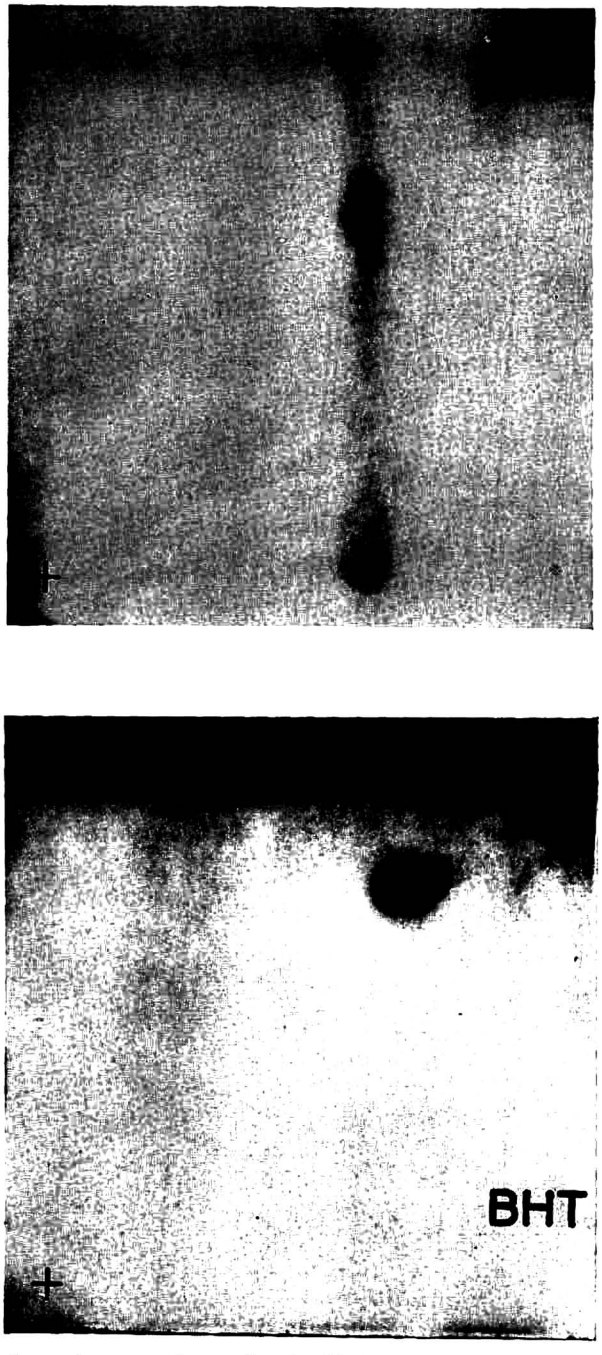


Fig. 2. Thin-layer chromatograms of egg phosphatidylethanolamines (170 μg aliquots) without and with BHT (0.1 %, w/v) in the solvent. Plates: Silica Gel G (250 μ), non-activated. Solvent: Chloroform–methanol–water (65:35:4, v/v/v) for both developments. First development, left to right. Plates left 20 h to dry. Second development, upwards. Plates dried 1 h at 80°. Detection, H_2SO_4.

METHODS FOR REMOVING THE ANTIOXIDANT

(1) *Vacuum oven.* When fractions are evaporated at 40° in a vacuum oven (15 mm Hg) BHT is lost with the eluents.

(2) *Vacuum desiccator.* Over silica gel (0.05 mm Hg) crystals of BHT disappear in a few hours, and solutions in lipids are completely purged within 24 h.

(3) *Chromatography.* A weak eluent such as carbon tetrachloride can be used to remove BHT from lipids on silicic acid. Small, wide columns permit rapid separations.

(4) *Solvent fractionation.*

(5) *Sublimation in a cold-finger apparatus.*

(6) *Steam distillation*[18].

(7) *Heating (thin-layer plates).*

PURIFICATION

Food grade BHT is purified before use by passage in carbon tetrachloride solution through activated silicic acid. M.p. 70.5–71°.

DISCUSSION

We now routinely add BHT to chromatographic solvents, usually at the 0.005 % level. It is easily detected and removed, although its presence is neglected for many purposes, *e.g.* deacylation[27] and acidic hydrolysis. It permits safe storage of lipid solutions almost indefinitely at —20°.

Use of BHT should greatly facilitate the application of preparative thin-layer chromatography to lipids.

ACKNOWLEDGEMENTS

We thank Miss M. MIDDLETON and Miss P. A. WATERS for preparing the photographs.

SUMMARY

Inclusion of small quantities of 4-methyl-2,6-di-*tert.*-butylphenol in solvents protects lipids from autoxidation during chromatography, manipulation, and storage. Since this compound has a higher chromatographic mobility than most lipids, it does not affect their separation. It is easily detected and removed when necessary.

REFERENCES

1 F. H. MATTSON AND R. A. VOLPENHEIN, *J. Lipid Res.*, 3 (1962) 281.
2 R. W. RIEMENSCHNEIDER, S. F. HERB AND P. L. NICHOLS, *J. Am. Oil Chemists' Soc.*, 26 (1949) 371.
3 C. H. LEA, in G. POPJÁK AND E. LE BRETON, *Biochemical Problems of Lipids*, Butterworths, London, 1956, p. 81.
4 G. ROUSER, A. J. BAUMAN, G. KRITCHEVSKY, D. HELLER AND J. S. O'BRIEN, *J. Am. Oil Chemists' Soc.*, 38 (1961) 544.
5 J. W. FARQUHAR, *Biochim. Biophys. Acta*, 60 (1962) 80.
6 G. H. DE HAAS, F. J. M. DAEMEN AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 65 (1962) 260.

[7] F. AYLWARD AND A. J. SHOWLER, *J. Sci. Food Agr.*, 13 (1962) 492.
[8] J. J. WREN, *J. Chromatog.*, 4 (1960) 173; *Chromatographic Reviews*, Vol. 3, Elsevier, Amsterdam, 1961, pp. 111, 177.
[9] G. H. STILLSON, D. W. SAWYER AND C. K. HUNT, *J. Am. Chem. Soc.*, 67 (1945) 303.
[10] L. HUNTER, *Progress in Stereochemistry*, Vol. 1, Butterworths, London, 1954, p. 223.
[11] H. R. GERSMANN AND A. F. BICKEL, *J. Chem. Soc.*, (1962) 2356.
[12] C. STAFFORD, *Anal. Chem.*, 34 (1962) 794.
[13] W. M. SMIT, *Anal. Chim. Acta*, 2 (1948) 671.
[14] L. J. BELLAMY AND R. L. WILLIAMS, *Proc. Roy. Soc.*, A 254 (1960) 119.
[15] J. J. WREN AND P. M. LENTHEN, *J. Chem. Soc.*, (1961) 2557.
[16] L. J. BELLAMY, G. EGLINTON AND J. F. MORMAN, *J. Chem. Soc.*, (1961) 4762.
[17] E. POTI, L. L. GENT, R. C. POMATTI AND H. LEVIN, *Anal. Chem.*, 25 (1953) 1461.
[18] C. R. SZALKOWSKI AND J. B. GARBER, *J. Agr. Food Chem.*, 10 (1962) 490.
[19] J. C. DACRE, *Biochem. J.*, 78 (1961) 758.
[20] A. SPORN AND O. SCHÖBESCH, *Igiena (Bucharest)*, 9 (1961) 113.
[21] M. G. DILLARD, A. S. HENICK AND R. B. KOCH, *J. Biol. Chem.*, 236 (1961) 37.
[22] I. E. BUSH, *The Chromatography of Steroids*, Pergamon, London, 1961, p. 349.
[23] J. J. WREN AND P. M. LENTHEN, *J. Chromatog.*, 5 (1961) 370.
[24] J. J. WREN, *Proc. 1st Intern. Congr. Food Science and Technol., London, 1962.*
[25] L. P. ZILL AND E. A. HARMON, *Biochim. Biophys. Acta*, 57 (1962) 573.
[26] F. J. M. DAEMEN, G. H. DE HAAS AND L. L. M. VAN DEENEN, *Rec. Trav. Chim.*, 81 (1962) 348.
[27] R. M. C. DAWSON, *Biochim. Biophys. Acta*, 14 (1954) 374.

CHARACTERISTICS OF SOLUTION FLOW IN SUPPORTING MEDIA IN THIN-LAYER ELECTROPHORESIS IN MOIST CHAMBERS

JERZY KOWALCZYK

Department of Technical Analytical Chemistry and Testing of Materials, Faculty of Chemistry, Technical University, Gdańsk (Poland)

(First received June 14th, 1963)
(Modified October 15th, 1963)

As is generally known the process of electrophoresis (ionophoresis) in supporting media is always associated with electro-osmotic movement. The solution movement is complicated only in moist chambers, as it is a resultant of two components, the electro-osmotic flow and the flow caused by water evaporation from the surface of the supporting media. MACHEBOEUF *et al.*[1,2] were the first to investigate this problem. Their experiments concerned the conditions of separation of the fractions of serum protein. Later investigations on solution flow were also carried out by DE WAEL[3], PEETERS AND VUYLSTEKE[4], AUDUBERT AND DE MENDE[5], SCHILLING AND WALDMANN-MAYER[6,7] and others. All the results obtained dealt with electrophoresis on filter paper. In the work described below experiments involving systems which do not contain filter paper were performed in moist chambers. We believe that the results of these investigations may be of interest as moist chambers are much used. In addition there has lately been considerable development of thin-layer electrophoresis and ionophoresis involving a variety of supporting media.

EXPERIMENTAL

Apparatus

A rectangular PCV wall chamber (25 × 10 × 10 cm), containing two 350 ml electrolyte vessels. Compact layers of cotton wool, rendering the flow of the products of electrolysis more difficult, were placed between the electrodes and the points of immersion of the electrophoretic media. The electrodes were made of platinum wire. The rectangular lid of the moist chamber was made of plexiglass.

Sources of voltage

(a) A d.c. valve rectifier of a stabilized output voltage range of 50–600 V and (b) an a.c. autotransformer of a stabilized output voltage range 0–250 V, 50 c/s.

Materials

(1) *Solutions*: 0.05 *N* hydrochloric acid; veronal-acetate buffer solution, pH = 8.6, prepared according to MICHAELIS[8].

(2) *Substances for supporting media*: Whatman No. 1 filter paper; glass fiber filter paper (60 × 80 threads of 0.3 mm gauge per cm^2); silica gel precipitated by the

method of HARNIS AND WICK[9]; alumina prepared from a commercial product according to ref. 10; titanium dioxide (E. Merck, Darmstadt); kaolin (Carter Products, Inc., New York, No. 606); starch (E. Merck, Darmstadt).

(3) *Solution flow indicator*: 1.5% glucose solution in 0.05 *N* hydrochloric acid or veronal-acetate buffer, pH = 8.6.

(4) *Reagent for development of the glucose spots*: 0.93 g aniline + 1.66 g phthalic acid and 100 ml of butyl alcohol[11].

Methods

(1) *Preparation of the layers of supporting media*: except for filter paper and glass fiber paper all the above substances were sieved and grains of maximum diameter of 0.06 mm were used. A glass plate measuring 15 × 5 cm and having a cavity of 2 mm depth was used. The cavity was filled with even wet layers of the substances. Pieces of Whatman No. 1 paper were attached to both ends of the layers. The plate with the layer thus prepared was immersed in the electrolyte solution and the chamber was closed hermetically and left overnight. The next day the excess of moist medium was removed from the plate surface leaving an evenly wet layer which measured exactly 15 × 5 × 0.2 cm^3.

(2) *Measurement of the solution flow velocities in the supporting media*: (a) Evaporation flow (Joule effect flow): by applying an alternating current, the effects of electro-osmosis were avoided and the flow obtained was due solely to evaporation of water from the medium surface. (b) Electro-osmotic flow: electro-osmotic flow velocities were calculated from the difference between the resultant flow velocity and the velocity of the flow occurring under the influence of water evaporation. (c) Resultant flow: on applying a direct current the solution flow obtained was that occurring normally in moist chambers as the result of electro-osmosis and evaporation.

Measurement of the fluid movement was carried out according to BERMES AND MCDONALD[12], using glucose as a neutral indicator independent of electric field influences. From the distance moved by the glucose it was possible to calculate the liquid velocities at various points along the supporting medium layers.

RESULTS AND DISCUSSION

Fig. 1 illustrates the effect of water evaporation on the solution flow. Results are shown for processes occurring under identical conditions (all supporting media had the same dimensions with the exception of filter paper layers and glass fiber paper). Considerable differences in flow velocities were evident although in these cases the current intensities were similar (*cf.* the current intensities for silica gel, alumina and titanium dioxide in Fig. 1). In addition, Fig. 1 shows that in each case described the solution flow conformed to the description of paper electrophoresis given by MACHEBOEUF[1,2]:

(a) the fluid movement was symmetrical to the centre of the layers,

(b) the straight line on the diagram characterized the velocities as a function of the distance from the beginning of the layers and

(c) the velocity was zero at the centre of the layers.

A subsequent determination of electro-osmotic flow velocities under the same conditions was attempted.

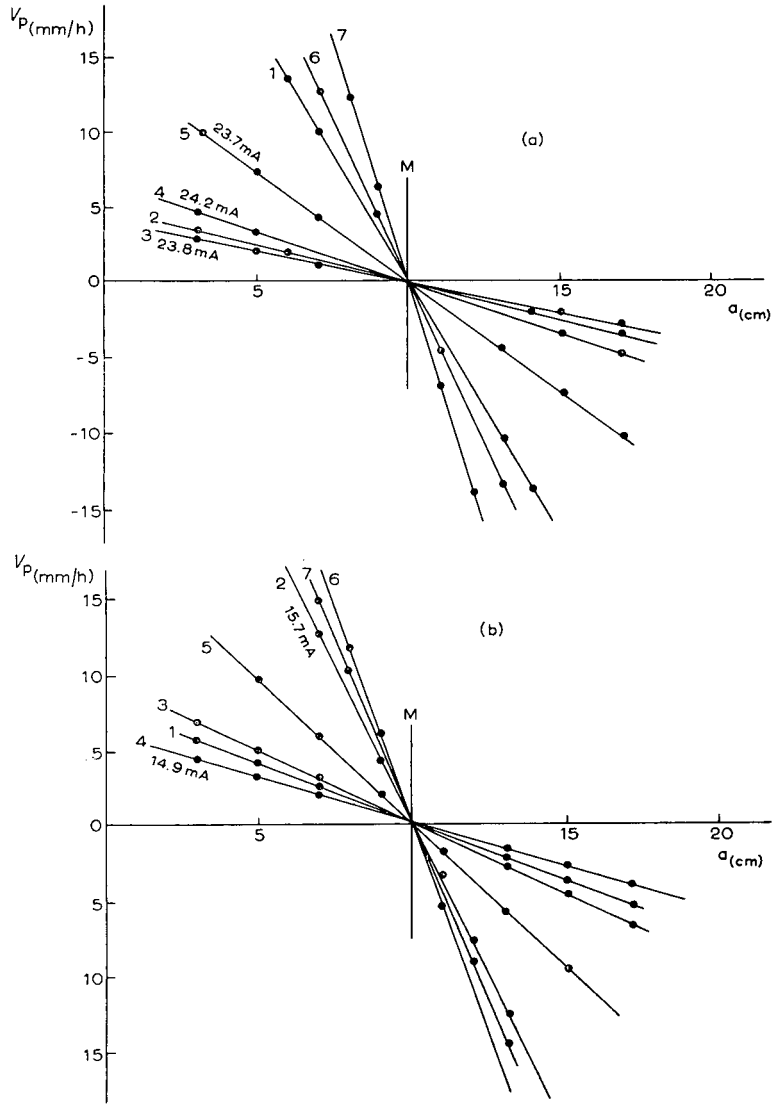


Fig. 1. Diagram of the solution flow, due to water evaporation. Curves of flow velocities, plotted as functions of the position on the medium layers. Solutions: Fig. a = 0.05 *N* hydrochloric acid; Fig. b = veronal-acetate buffer pH = 8.6. Supporting media: 1 = starch; 2 = titanium dioxide; 3 = alumina; 4 = silica gel; 5 = kaolin; 6 = glass fiber filter paper; 7 = Whatman No. 1 filter paper. Voltage 7.5 V/cm, a.c. 50 c/s. Time = 60 min.

Fig. 2 shows the results of the calculations.

The magnitude and direction of the electro-osmotic movement show considerable differences on the above mentioned media. As was expected in these cases, the liquid flowed with a constant velocity along the layers. The resultant solution flow is shown in Fig. 3.

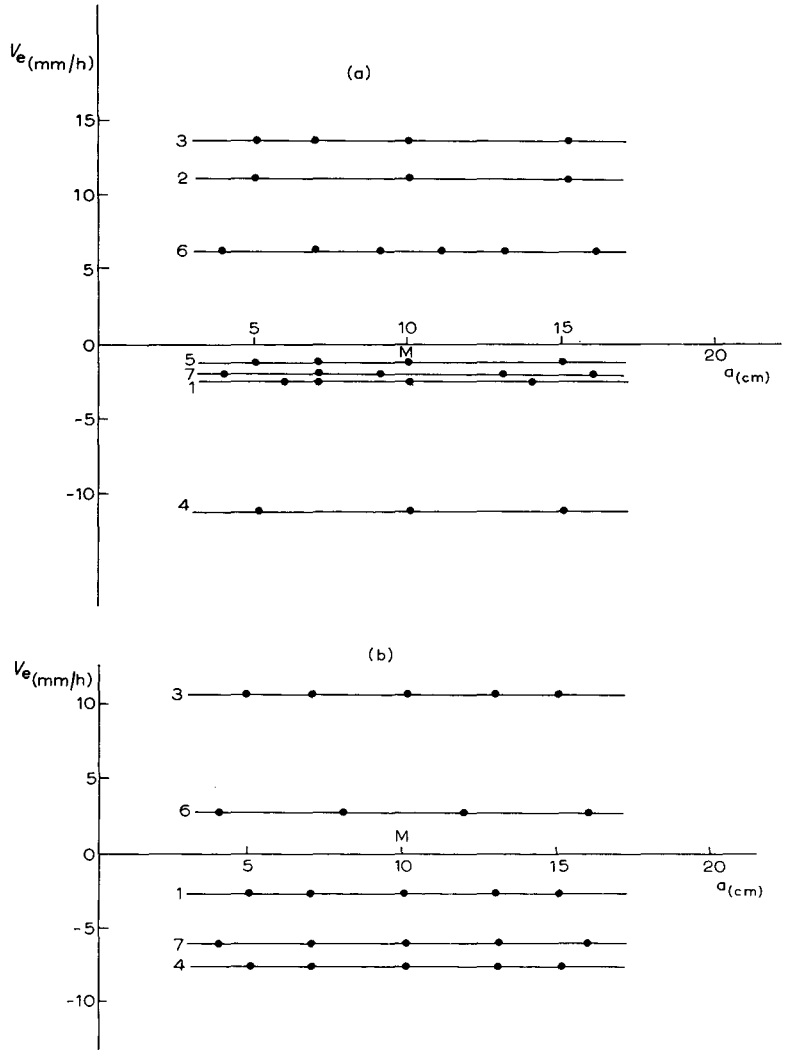


Fig. 2. Diagram of electroosmotic flow. Curves of solution flow velocities plotted as functions of position on the medium layers. Solutions: Fig. a = 0.05 *N* hydrochloric acid; Fig. b = veronal-acetate buffer pH = 8.6. Supporting media: 1 = starch; 2 = titanium dioxide; 3 = alumina; 4 = silica gel; 5 = kaolin; 6 = glass fiber filter paper; 7 = Whatman No. 1 filter paper. Voltage = 7.5 V/cm, d.c. Time = 60 min.

Figs. 3a and 3b show the cases where a point of hydrostatic equilibrium[1,2], π, on the supporting medium exists. In this zone π, the resultant fluid motion equalled zero. The curves in Figs. 3c and 3d show on the other hand the solution flow in cases where there was no point π on the supporting media (π being a hypothetical zone lying beyond the medium layers). The results obtained in Fig. 3 are clear, taking into consideration the differences in the values obtained for both flow components illustrated in Figs. 1 and 2.

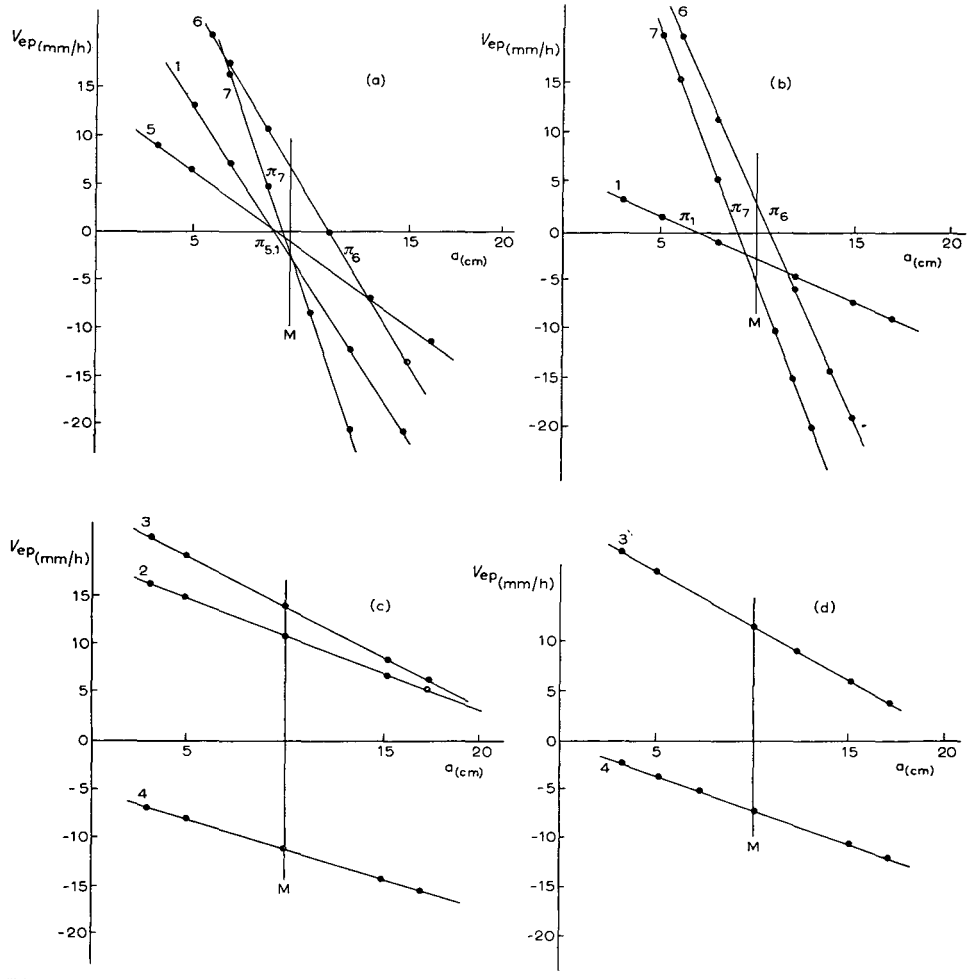


Fig. 3. Diagram of resultant solution flow in supporting media during electrophoresis in moist chambers. Curves describing flow velocities as function of position along the medium layers. Figs. a and b = systems in which there is a zone of hydrostatic equilibrium π, where the liquid velocity equals zero. Figs. c and d = systems in which there is no zone π. Solutions: Figs. a and c = 0.05 *N* hydrochloric acid, Figs. b and d = veronal acetate buffer pH = 8.6. Supporting media: 1 = starch; 2 = titanium dioxide; 3 = alumina; 4 = silica gel; 5 = kaolin; 6 = glass fiber filter paper; 7 = Whatman No. 1 filter paper. Voltage = 7.5 V/cm, d.c. Time = 60 min.

CONCLUSIONS

Two groups of systems, supporting medium + solution, exist under the conditions usually present in electrophoresis (ionophoresis) in moist chambers. They are: (1) systems in which a zone of hydrostatic equilibrium, π, exists in the medium layer and (2) systems in which there is no zone π.

As can be seen in Fig. 3, in systems of the first kind the solutions flow in a variable and complicated way from both electrolyte vessels to the zone π. In systems without a zone π, the solution flow, being unidirectional and uniformly increasing, is less

complicated. Furthermore, after some time, changes of electrolyte concentrations occur at the zone π. This fact has already been stated by some authors in the case of filter paper[1,2,6,7]. On this basis, one may deduce the existence of more stable and more easily defined conditions for electrophoresis in moist chambers in supporting media having no zone π.

It may be interesting to note that we used filter paper as our supporting medium. As this is a system with zone π, it is therefore one of the less advantageous systems. Further investigations on this subject will be continued and described in subsequent publications[13,14].

SUMMARY

Solution flows during thin-layer electrophoresis in moist chambers, for seven supporting media, were characterized. Two groups of systems, supporting medium + solution, were defined. They are: (1) systems with a zone of hydrostatic equilibrium, π, in the medium layer, (2) systems having no zone π. It may be deduced that the second group of systems is more advantageous in practice.

REFERENCES

1 M. MACHEBOEUF, P. REBEYROTTE, J. M. DUBERT AND M. BRUNERIE, *Bull. Soc. Chim. Biol.*, 35 (1953) 334.
2 M. MACHEBOEUF, J. M. DUBERT AND P. REBEYROTTE, *Bull. Soc. Chim. Biol.*, 35 (1953) 346.
3 J. DE WAEL, *CIBA Foundation Symposium on Paper Electrophoresis*, J. & A. Churchill, Ltd., London, 1956.
4 H. PEETERS AND P. VUYLSTEKE, *Colloq. St. Jans. Hosp., Brugge, Belg., 4me*, De Tempel, Brugge, 1956.
5 R. AUDUBERT AND S. DE MENDE, *The Principles of Electrophoresis*, Hutchinson, London, 1959.
6 K. SCHILLING AND H. WALDMANN-MEYER, *Acta Chem. Scand.*, 13 (1959) 1.
7 H. WALDMANN-MEYER, *Chromatographic Reviews 5*, Elsevier, Amsterdam, 1963, pp. 1–45.
8 L. MICHAELIS, *Biochem. Z.*, 234 (1931) 139.
9 R. HARMIS AND A. N. WICK, *Ind. Eng. Chem., Anal. Ed.*, 18 (1946) 276.
10 E. LEDERER AND M. LEDERER, *Chromatography*, 2nd Ed., Elsevier, Amsterdam, 1957, p. 24.
11 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press, New-York, 1958.
12 E. W. BERMES AND H. J. MCDONALD, *J. Chromatog.*, 4 (1960) 34.
13 J. KOWALCZYK, *Chem. Anal. (Warsaw)*, 8 (1963) 659, 823, 835; 9 (1964) 21, 29.
14 J. KOWALCZYK, in the press.

THIN-LAYER CHROMATOGRAPHY OF PRESERVING AGENTS

J. W. COPIUS-PEEREBOOM AND HENNY W. BEEKES
*Government Dairy Station, Leiden (The Netherlands)**

(Received August 16th, 1963)

At the present time, the use of substances with preservative properties is wide-spread in food technology. In many countries the addition of preservatives is now regulated by food legislation. Benzoic acid, sorbic acid, the ethyl and propyl esters of *p*-hydroxybenzoic acid, and the corresponding sodium salts, are permitted in most European countries. Reliable methods for the analysis of these preserving agents, preferably by chromatography, are therefore of prime importance to laboratories working in this field.

Many paper chromatographic systems have been devised for the purpose. A suitable system was described by JARCZYNSKI[1], using the upper layer of the mixture *n*-butanol–35% ammonia–water (70:20:10), to which 2.5% of 96% ethanol is added. In the Netherlands many laboratories are using this procedure, *vide* CATS[2]. However, the time-consuming character of the paper chromatographic analysis may sometimes present difficulties. Furthermore, few colour reactions specific for the various types of preservatives have been described in the literature**.

The technique of thin-layer chromatography (T.L.C.) may be advantageous especially since more aggressive reagents can be applied on chromatoplates and the analysis time may be decreased considerably.

Separation of the methyl, ethyl, propyl, and *n*-butyl esters of *p*-hydroxybenzoic acid has been described by GÄNSHIRT[3]. Kieselgel G-coated chromatoplates were strongly activated (2 h at 160°) and developed in the solvent mixture pentane–acetic acid (8:2). The four esters are completely fractionated. However, we did not succeed in separating benzoic acid and sorbic acid either on normally or on strongly activated kieselgel G plates.

At the time of this investigation no other papers dealing with T.L.C. of preservatives had come to our notice.

CELLULOSE CHROMATOPLATES

To begin with, we investigated the migration rates of benzoic acid and sorbic acid in several polar solvent mixtures using chromatoplates coated with a cellulose layer (cellulose powder MN 300, gypsum-free). The best separation, comparatively, of

* Rijkszuivelstation, Vreewijkstraat 12B, Leiden.

** In our laboratory we are accustomed to use a procedure of developing the spots of sorbic acid and benzoic acid on paper strips. Successive spraying with a bromophenol blue–methyl red mixture and with potassium permanganate yields yellow-green and purple spots respectively. The sorbic acid spot may be detected specifically by spraying first with a 10% potassium dichromate solution and afterwards with a saturated thiobarbituric acid solution. Sorbic acid is revealed by a bright red coloured spot.

these two preservatives was obtained in the *n*-butanol–ammonia–water (70:20:10) mixture described by JARCZYNSKI, but in this system the development procedure still takes about 5–6 h. The degree of separation of benzoic acid and sorbic acid is only slightly better than when paper strips are used (see Fig. 1). The R_F values of several preservatives in this system are given in Table I.

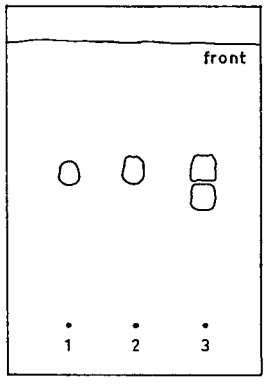


Fig. 1. Separation of preservatives on cellulose plates in *n*-butanol–35% ammonia–water (70:20 10). Spotted amount = 100 μg. Detection: bromophenol blue, followed by potassium permanganate. Spot 1 = salicylic acid. Spot 2 = sorbic acid. Spot 3 = a benzoic acid–sorbic acid mixture (1:1).

In this system the methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid are completely separated.

The spots are developed by spraying successively with a bromophenol blue–methyl red mixture and with potassium permanganate[1].

The cellulose plate is dried thorougly in air (with care on account of acid vapour). Bromophenol blue (120 mg) is dissolved in 100 ml of water and 60 mg of methyl red in 100 ml of 96% ethanol. The two solutions are mixed and 100 ml of phosphate buffer (pH = 7.17) is added. The plate is sprayed intensively with this mixture and

TABLE I

R_F AND R_S* VALUES OF PRESERVATIVES ON CELLULOSE CHROMATOPLATES IN *n*-BUTANOL–35% AMMONIA–WATER (70:20:10)

"Kammerübersättigung". Spotted amount = 100 μg.

	R_F value	*R_S value*
Benzoic acid	0.50	0.91
Sorbic acid	0.58	1.07
Salicylic acid	0.56	≡1.0
Dehydroacetic acid	0.09	0.16
p-Hydroxybenzoic acid	0.09	0.16
Methyl *p*-hydroxybenzoate	0.75	1.42
Ethyl *p*-hydroxybenzoate	0.86	1.62
Propyl *p*-hydroxybenzoate	0.90	1.70
o-Phenylphenol	0.95	1.61

* *S* = salicylic acid.

afterwards with a solution of potassium permanganate (0.5 %) and sodium carbonate (1 %) (see Table III, reagent 6).

At first the spot of benzoic acid attains a violet colour, but after some time it becomes quite colourless. Upon spraying, sorbic acid exhibits a green colour that changes quickly into a stable purple colour (on a light blue background).

KIESELGEL–KIESELGUR PLATES

Testing several types of adsorbents and mobile phases—among others the system of GÄNSHIRT[3] and the systems of PETROWITZ[4] and BRAUN[5] devised for the separation of polyfunctional acids—we were not able to obtain a separation of benzoic acid and sorbic acid. However, we found that a mixture of equal amounts of kieselgel G–kieselgur G (both of Merck) was suitable for the separation of these preservatives. Especially when using the solvent mixture hexane–acetic acid (96:4) under "Kammerübersättigung"-conditions a distinct separation of benzoic acid and sorbic acid was obtained. With this adsorbent mixture (15 g of adsorbent mixed with 32 ml of water) layers of about 0.25 mm are prepared. The amount of preservative used for spotting is 50 μg and the length of the run is about 20 cm (in 1.5 h). The R_F and R_S (S = salicylic acid) values of some preservatives in this system are given in Table II.

The esters of *p*-hydroxybenzoic acid were not resolved under these circumstances.

TABLE II

R_S (S = SALICYLIC ACID) VALUES OF PRESERVATIVES ON KIESELGEL G–KIESELGUR G (1:1) PLATES IN THE SOLVENT MIXTURES HEXANE–ACETIC ACID (96:4), A, AND PETROLEUM ETHER–ETHER–ACETIC ACID (80:20:1), B.

Spotted amount = 50 μg. Detection: Ultraphor W.T. and Rhodamine B.

	A	*B*
Benzoic acid	1.54	1.11
Sorbic acid	1.28	0.91
Salicylic acid	≡1.00	≡1.00
Dehydroacetic acid	0.60	0.88
p-Hydroxybenzoic acid	0.07	0.41
Methyl *p*-hydroxybenzoate	0.12	0.75
Ethyl *p*-hydroxybenzoate	0.16	0.79
Propyl *p*-hydroxybenzoate	0.18	0.84
o-Phenylphenol	1.13	1.36

SPOT DEVELOPMENT

The spots of the preservatives are developed by admixing 0.02 % of the fluorescence indicator Ultraphor W.T. (B.A.S.F.) to the adsorbent–water mixture and viewing the chromatoplate under 366 nm radiation (COPIUS-PEEREBOOM[6]). Alternatively, 2 % of "Leuchtpigment ZS-Super" (RIEDEL DE HAAN) is admixed to the adsorbent mixture and the plates are viewed in 254 nm radiation (GÄNSHIRT[3]).

We prefer, however, to use more specific colour reactions which may provide some valuable information as to the identity of unknown preservatives, isolated from food products.

TABLE

COLOUR REACTIONS OF SOME PRESERVATIVES
KIESELGEL G–KIESELGUR

Compound	1	2	3	4	5	
	Rhodamine B–H_2O_2 366 nm	*Bromocresol green*	*Thiobarbituric acid*	*Thymol*	*Successive H_2O_2, $FeCl_3$ sprays*	
					A	*B*
Salicylic acid	purple	orange-yellow	faint purple	—	purple	purple
Benzoic acid	dark purple	yellow	—	—	faint purple (with yellow centre)	*dark brown*
Sorbic acid	pink	yellow	*bright red*	*purple*	faint yellow	—
o-Phenylphenol	purple	—	—	—	—	violet
Dehydroacetic acid	purple	yellow	—	—	yellow-brown	orange-brown
p-Hydroxybenzoic acid	blue	yellow	—	yellow	yellow-brown	green
Methyl *p*-hydroxybenzoate	pink	—	—	yellow	—	gray
Ethyl *p*-hydroxybenzoate	pink	—	—	yellow	—	gray
Propyl *p*-hydroxybenzoate	pink	—	—	yellow	—	gray

After the development procedure, the plate is dried and then intensively sprayed with a 0.05 % Rhodamine B solution. The spot of benzoic acid is coloured violet to purple, while the sorbic acid spot attains a more pink hue. The fluorescence of both spots under 366 nm radiation shows still greater differences. Benzoic acid exhibits a dark purple-blue fluorescence and sorbic acid only a light orange-pink one. These characteristic differences are enhanced by spraying afterwards with a 3 % H_2O_2 solution (Table III, reagent No. 1). The spots have to be marked directly after the spraying procedure, because they tend to diffuse after a time. Both detection procedures, *viz.* 0.02 % of Ultraphor in the adsorbent layer and spraying with the Rhodamine B–H_2O_2 solutions, can be combined advantageously, yielding a strong blue fluorescence of the benzoic acid and a pink one of the sorbic acid spot (366 nm radiation). Of course other acid–base indicators can be used in the same way, *e.g.* bromocresol green (see Fig. 2 and Table III, reagent No. 2).

The spots of benzoic acid and of sorbic acid are clearly separated after one single development. When Rhodamine B is used, the degree of separation of these preservatives is decreased after two or three developments, but when Ultraphor is added to the adsorbent layer a multiple development may be applied.

Analysis of extracts isolated from various types of food products by means of specific colour reagents should give valuable evidence concerning the identity of the preservatives present.

For that purpose we have elaborated several characteristic colour reactions (*vide*

III

(SPOTTED AMOUNTS 100 μg) ON G CHROMATOPLATES

6	7	8	9	10	11	12
Bromophenol blue–$KMnO_4$	*Bromophenol blue –methyl red + Pauly's reagent*	*D.Q.C.*	*p-Nitro-aniline*	*Ceric sulphate*	*$TiCl_3$*	*$FeCl_3$*
yellow	faint yellow	—	—	brown-gray	yellow	purple
faint yellow	—	—	—	—	—	—
yellow	faint yellow	—	—	brown	—	faint yellow
purple	*strong yellow*	*orange-brown*	*brown-red*	*violet*	—	—
yellow	—	gray	—	—	*blue-purple*	brown-yellow
yellow	yellow	yellow	—	faint purple	—	yellow-brown
yellow	—	faint yellow	—	faint yellow	—	—
yellow	—	faint yellow	—	faint yellow	—	—
yellow	—	faint yellow	—	faint yellow	—	—

Table III). Thus, *sorbic acid* is detected specifically by spraying with a saturated solution of thiobarbituric acid (Table III, reagent no. 3). The spot of sorbic acid is revealed by a bright red colour, while most of the other preservatives are not coloured at all. In another colour reaction the plate is sprayed with a 20 % ethanolic thymol solution, heated 10 min at 90°, sprayed with 4*N* sulphuric acid, and finally heated 10 min at 120° (reagent No. 4). Sorbic acid is then revealed by a blue-purple spot.

The presence of *benzoic acid* on a chromatoplate is determined as follows: The dried chromatoplate is sprayed with a 3 % H_2O_2 solution, heated 5 min at 90°, cooled and sprayed with a 2 % ferric(III)chloride solution (reagent no. 5A). The spot of benzoic acid attains a faint purple hue, while sorbic acid is coloured faintly yellow. The plate is heated 5 min at 90°, sprayed again with 2 % ferric(III)chloride and afterwards with 3 % H_2O_2. The colour of the sorbic acid spot now fades away quickly, whereas benzoic acid is at first revealed as a white spot on a yellowish background. After some time (about 1 h) benzoic acid attains a dark brown colour, while the sorbic acid spot has become completely colourless (reagent No. 5B).

The spot of *o-phenylphenol* can be revealed by several specific reagents *viz.* (a) bromophenol blue–methyl red and PAULY's reagent, (b) dichlorobenzoquinone chlorimine (D.Q.C.), (c) *p*-nitroaniline, and (d) ceric(IV)sulphate.

(a) The plate is sprayed with the bromophenol blue–methyl red reagent mentioned above (see cellulose plates) followed by PAULY's reagent (diazotized sulphanilic acid). The spot of *o*-phenylphenol is coloured bright yellow (reagent No. 7).

(b) The plate is sprayed with a 1 % solution of 2,6-dichloro-*p*-benzoquinone-4-chlorimine (reagent No. 8), dried and sprayed with a 2 % borax solution (in 40 % ethanol). The *o*-phenylphenol spot is revealed by an orange-brown colour.

(c) The plate is sprayed with a solution of diazotized *p*-nitroaniline (reagent No. 9). The spot of *o*-phenylphenol has a brown-red colour.

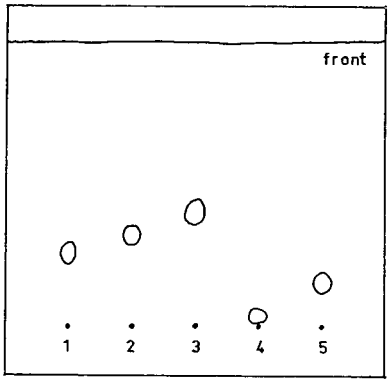


Fig. 2. T.L.C. of preservatives on kieselgel G–kieselgur G plates using the mobile phase hexane–acetic acid (96:4). Spotted amount = 100 μg. Detection: bromocresol green (0.04 % in 96 % ethanol with addition of potassium hydroxide till green, *vide* Table III, reagent No. 2). Spot 1 = salicylic acid. Spot 2 = sorbic acid. Spot 3 = benzoic acid. Spot 4 = *p*-hydroxybenzoic acid. Spot 5 = dehydroacetic acid.

(d) Trichloroacetic acid (1 g) is dissolved in 4 ml of a 2.5 % solution of ceric(IV) sulphate and heated. Concentrated sulphuric acid is added until the solution remains clear. When sprayed with this mixture (reagent No. 10), the *o*-phenylphenol attains a violet colour.

The presence of *dehydroacetic acid* can be detected by spraying the chromatoplate with a 3 % titanium(III)chloride solution (reagent No. 11). Salicylic acid attains a yellow colour, dehydroacetic acid a purple-blue one.

The spot of *salicylic acid* is revealed specifically on Ultraphor-free chromatoplates by a bright fluorescence under 366 nm radiation and by a purple colour when sprayed with a 2 % ferric(III) chloride solution (reagent No. 12).

The colours in the above reactions given by a number of preservatives are listed in Table III.

Many other solvent systems were tested, but in none of them could benzoic acid and sorbic acid be separated distinctly. A comparatively good separation was also achieved using the solvent mixture petroleum ether–ether–acetic acid (80:20:1), as is shown in Table II. In that system the spots of benzoic acid and sorbic acid were separated after three successive developments (detection: Ultraphor W.T.). Salicylic acid and benzoic acid could not, however, be resolved under these conditions.

Applying the kieselgel G–kieselgur G/hexane–acetic acid (96:4) system and selecting the most appropriate colour reactions, the identity of a preservative can be definitely determined. The procedures described above were amply tested in practice, especially in the analysis of preservatives isolated from food products such as margarines, packaging materials and the plastic dispersions used as cheese coating.

KIESELGEL PAPER

We have succeeded in performing analogous separations on commercial Schleicher and Schüll, no. 289 kieselgel papers. The *p*-hydroxybenzoic acid esters are separated in the solvent mixture hexane–acetic acid (96:4), whereas the separation of benzoic acid and sorbic acid can be accomplished with hexane–acetic acid (99.8:0.2). The preservative spots are developed on the kieselgel paper by spraying with 0.05 % Rhodamine B or with 0.5 % quinine bisulphate (*vide* Fig. 3).

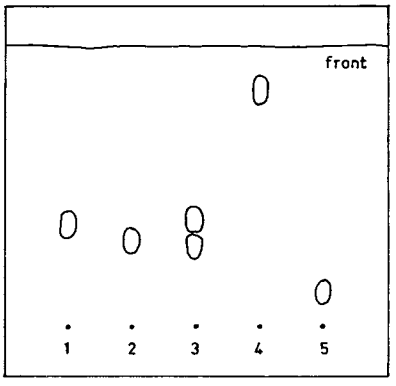


Fig. 3. Separation of preservatives on Schleicher and Schüll, No. 289 kieselgel paper with the mixture hexane–acetic acid (99.8:0.2). Spotted amounts = 80 μg. Detection: 0.05 % of Rhodamine B. Spot 1 = benzoic acid. Spot 2 = sorbic acid. Spot 3 = mixture 1 + 2. Spot 4 = *o*-phenylphenol. Spot 5 = dehydroacetic acid.

ACKNOWLEDGEMENT

The authors would like to express their gratitude to Dr. J. G. VAN GINKEL, Director of the Rijkszuivelstation (Government Dairy Station) for his permission to develop these methods and to publish the results in this paper.

SUMMARY

The separation of preserving agents and especially of benzoic acid and sorbic acid by thin-layer chromatography was studied. Both preservatives can be separated on cellulose chromatoplates using the upper phase of the solvent mixture *n*-butanol–35 % ammonia–water (70:20:10). Still better and much more rapid separations are obtained on chromatoplates coated with a kieselgel G–kieselgur G (1:1) adsorbent mixture, especially when developing the plate with the mobile phase hexane–acetic acid (96:4).

Several specific colour reactions are described which can be applied in the analysis of the preservatives isolated from food products, packaging material etc.

REFERENCES

1 R. JARCZYNSKI AND F. KIERMEIER, *Z. Lebensm. Untersuch. -Forsch.*, 99 (1954) 91.
2 H. CATS AND H. ONRUST, *Chem. Weekblad*, 54 (1958) 456.
3 H. GÄNSHIRT AND K. MORIANZ, *Arch. Pharm.*, 293 (1960) 1065.
4 H. J. PETROWITZ AND G. PASTUSKA, *J. Chromatog.*, 7 (1962) 128.
5 D. BRAUN AND H. GEENEN, *J. Chromatog.*, 7 (1962) 56.
6 J. W. COPIUS-PEEREBOOM, *J. Chromatog.*, 4 (1960) 323.

CHROMATOGRAPHIC SEPARATION OF C_{19}-16-DEHYDRO-STEROIDS

D. B. GOWER

Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London (Great Britain)

(First received July 23rd, 1963)
(Modified October 17th, 1963)

Since the discovery of androst-16-en-3α-ol in male and female urine by BROOKSBANK AND HASLEWOOD[1] and of its biosynthesis in testis and adrenocortical tissue by GOWER AND HASLEWOOD[2] and GOWER[3], the need for chromatographic systems to separate this compound from other C_{19}-16-dehydro-steroids which might also occur naturally has become increasingly acute. Besides being all weakly polar, these compounds which have been used in the present study, only differ from one another by changes in configuration at the A/B ring junction or by one double bond, thus making their separation more difficult. This difficulty has already been appreciated by BAKER AND GOWER[4], when only partial success was obtained with gas chromatography using a silicone gum stationary phase. Column chromatography with Al_2O_3 as adsorbent has been employed for the initial purification of urinary or tissue extracts and separates androst-16-en-3α-ol but the group of closely related steroids, called the "androstenol analogues" by BROOKSBANK[5] and by GOWER[3], are eluted in one fraction.

In view of the success with which thin layer chromatography has been employed in the separation of weakly polar steroids by BARBIER, JÄGER, TOBIAS AND WYSS[6], by VAN DAM, DE KLEUVER AND DE HEUS[7] and by VAN DAM[8], this technique has been applied to the present problem. In addition, an investigation has been made of some of the paper chromatographic systems suggested by other workers for the separation of weakly polar steroids employing as stationary phase phenylcellosolve (NEHER AND WETTSTEIN[9]; RUBIN, DORFMAN AND PINCUS[10]), kerosene (MARTIN[11]) and liquid paraffin (KODICEK AND ASHBY[12]). Systems using papers which had been fully acetylated (RITTER AND HARTEL[13]) or impregnated with silicic acid (LEA, RHODES AND STOLL[14]) have also been investigated.

EXPERIMENTAL

The 16-dehydro steroids used in this study were 5α-androst-16-en-3α-ol (An (α)), 5α-androst-16-en-3β-ol (An (β)), 5β-androst-16-en-3α-ol (aetiochol-16-en-3α-ol, Ae), androsta-5,16-dien-3β-ol (Andien). Oestra-1,3,5(10),16-tetraen-3-ol (Oe), a C_{18} steroid, was used as a comparison. An (α) was prepared by BROOKSBANK AND HASLEWOOD[1] and the other compounds were generously supplied by Dr. C. L. Hewett of Organon Ltd. Acetates were prepared using pyridine and acetic anhydride at room temperature overnight and were recrystallised from aqueous ethanol. Solutions for chromatography of concentrations of about 1 mg/ml and 10 mg/ml were made in methanol.

Thin layer chromatography

Smooth glass plates (18 × 18 cm or 18 × 6 cm, thickness 0.4 cm) were normally used although, in preliminary experiments, the use of microscope slides allowed a large number of runs to be performed in a short time. Initially, plates were covered with Kieselgel G using a thin-layer applier[15] but since unevenness and slight irregularities of the layer were not found to influence greatly the R_F of the same compound applied at intervals across a plate, the spreading was performed by hand in later experiments. In this case, a fairly thin mixture of one part Kieselgel G to three parts of water was prepared. The consistency was such that the mixture could be poured on to the plates after which the latter were gently tilted so as to allow fairly uniform spreading. Plates so prepared were dried first in air for 30 min and then at 100° for 60 min. The initial slow drying process appeared to be essential to prevent cracking of the surface of the thin-layer during reactivation at 100°. The preparation and development of chromatostrips and chromatoplates using the ascending technique were as described previously[7].

On some occasions plates were re-run. When the solvent front had almost reached the end of the plate, it was removed from the solvent and the position of the front marked. The plate was then dried, replaced in the solvent and the vessel closed. When the solvent front had reached the pre-marked position, the plate was again removed and dried. This procedure was repeated three or four times, to allow an effective run of solvent of about 50 cm.

Paper chromatography

Whatman No. 3MM paper was impregnated with silicic acid by the method of LEA, RHODES AND STOLL[14]. Runs were performed using the ascending technique by dipping one end of the paper into a few cm depth of solvent contained in a glass jar or tank which was then closed. No period of equilibration was necessary.

Detection of steroids

The colour reaction of BROOKSBANK AND HASLEWOOD[1] has been used to detect the 16-dehydro-steroids and their acetates used in this study on chromatostrips, chromatoplates and on some paper chromatograms. The two reagents (resorcyaldehyde in glacial acetic acid (0.5% w/v) and concentrated sulphuric acid in glacial acetic acid (5% v/v) were mixed in equal volumes prior to spraying. This solution will be abbreviated to RA. Colour development at 100°, however, was largely inhibited by the presence of non-volatile, stationary phases remaining on paper chromatograms after drying and, moreover, the reagent could not be used on fully acetylated paper since the latter was attacked by sulphuric acid. Of more general use, therefore, was the phosphomolybdic acid reaction (PMA) of KRITCHEVSKY AND KIRK[16] although the presence of large quantities of phenylcellosolve partially inhibited colour development. The use of phosphotungstic acid (PTA) in ethanol[11], iodine in light petroleum[17] and ALLEN[18] reagent (concentrated sulphuric acid–ethyl alcohol–water (80:18:2, by vol.)) has also been investigated. A solution of uranyl nitrate (5% w/v) in 10% v/v aqueous sulphuric acid* has also been used to detect the steroids on plates although silicic acid-impregnated paper charred with it on heating.

* I am grateful to Dr. R. J. BRIDGWATER for drawing my attention to this reagent.

TABLE I

APPROXIMATE R_F VALUES OF SOME 16-DEHYDRO-STEROIDS AND THEIR ACETATES ON KIESELGEL G IN VARIOUS SOLVENTS AND SOLVENT MIXTURES

*Compound**	*Solvents***												*Colours with visualising reagents****				
	10	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*PMA**	*PTA**	*RA**	*Allen*[20]	*Uranyl nitrate*
An (α)	0.08	0.29	0.21	0.34	0.24	0.33	0.66	0.40	0.75	0.61	0.74	0.52	bg	br	m	gr	gr
Ae	0.05	0.22	0.19	0.26	0.20	0.27	0.58	0.33	0.62	0.55	0.65	0.45	g	br	m	gr	bgr
An (β)	0.04	0.19	0.15	0.20	0.15	0.20	0.48	0.27	0.46	0.45	0.58	0.38	b	br	m	gr	p
Andien	0.04	0.18	0.16	0.23	0.17	0.23	0.49	0.28	0.49	0.47	0.59	0.40	p	ro	b	m	p
Oe	0.15	0.38	0.37	0.48	0.30	0.51	0.72	0.51	0.93	0.77	0.88	0.80	rp	o	r	pk	r
An (α) acetate	0.34	0.69	0.51	0.64	0.75	0.91	1.0	0.68	1.0	—	0.87	0.81	g	ybr	m	gr	pk
Ae acetate	0.36	0.75	0.52	0.63	0.74	0.93	1.0	0.70	1.0	0.85	0.87	0.81	g	ybr	m	gr	bgr
An (β) acetate	0.40	0.88	0.54	0.62	0.77	0.91	1.0	0.71	1.0	0.91	0.88	0.78	g	ybr	m	gr	p
Andien acetate	0.40	0.79	0.55	0.61	0.79	0.89	1.0	0.71	1.0	0.84	0.89	0.75	p	p	m	m	br
Oe acetate	0.43	0.79	0.53	0.64	0.80	0.94	1.0	0.72	1.0	0.85	0.87	0.78	m	or	r	pk	r

* For abbreviations see Experimental Section.

** Solvents; abbreviations: 10 = toluene; 11 = methylene chloride; 12 = toluene–ethyl acetate, 19:1 (v/v); 13 = toluene–ethyl acetate, 9:1 (v/v); 14 = benzene–ethanol, 98:2 (v/v); 15 = benzene–ether, 9:1 (v/v); 16 = benzene–acetone, 85:15 (v/v); 17 = benzene–methyl ethyl ketone, 9:1 (v/v); 18 = methylene chloride–ethyl acetate, 9:1 (v/v); 19 = ethylene chloride–ethyl acetate, 90:12 (v/v); 20 = di-isopropyl ether–acetic acid, 96:4 (v/v); 21 = di-isopropyl ether–formic acid, 99:1 (v/v).

*** Colours; abbreviations: b = blue; bg = bluish green; bgr = bluish grey; br = brown; g = green; gr = grey; m = mauve; o = orange; r = red; or = orange red; ro = rose; rp = reddish purple; p = purple; pk = pink; y = yellow; yb = yellow brown.

RESULTS

Thin layer chromatography

The R_F values of An (α) and other 16-dehydro-steroids have been determined in a number of solvents and solvent mixtures using Kieselgel G as adsorbent. These data, together with those obtained for the acetates, are given in Table I. An (α), Ae and Oe could be separated from each other and from An (β) and Andien in a number of systems. The latter pair of compounds, however, resisted all attempts at resolution. The effect on the resolution of this pair of compounds of re-running some systems up to three or four times was studied. Using this technique a separation sufficient for their identification in a mixture has been achieved. Differences in the mobility of An (α) and Ae have also been accentuated (Table II). It appeared that an effective

TABLE II

EFFECT OF RE-RUNNING ON THE SEPARATION OF SOME 16-DEHYDRO-STEROIDS

Solvent	*Effective distance moved by solvent front (cm)**	*Distance moved from origin by steroids** (cm)*				
		An (α)	*Ae*	*An (β)*	*Andien*	*Oe*
Benzene–methyl ethyl ketone, 9:1 (v/v)	15.0	6.0	4.95	4.1	4.2	7.65
	44.7	10.75	9.65	8.4	8.7	12.4
	64.0	12.0	11.0	9.5	10.1	14.2
Toluene–methyl ethyl ketone, 9:1 (v/v)	44.4	9.25	7.95	6.5	6.85	11.3
	58.0	11.2	10.15	8.75	9.0	12.8
Toluene–ethyl acetate, 9:1 (v/v)	15.6	5.2	4.1	3.25	3.55	7.4
	45.9	8.7	7.4	6.15	6.55	11.65
	65.6	10.5	9.15	7.35	7.75	13.55
Ethylene chloride–ethyl acetate, 90:12 (v/v)	14.7	8.9	8.1	6.85	7.25	11.3
	30.6	12.0	10.9	9.1	9.6	13.8

* Chromatography was performed on glass plates (18 cm × 18 cm) covered with Kieselgel G. Development was continued until the solvent front had reached a pre-marked line. The plate was then removed from the solvent, dried and re-run. This procedure was repeated until the solvent front had effectively moved three to four times the length of the plate.

** For abbreviations, see Experimental Section.

run of 40–50 cm gave the maximum resolution possible in, for example, benzene–methyl ethyl ketone (9:1, v/v) and that there was little advantage to be gained from further runs. A typical run for the solvent to move an effective distance of 3 × 16 cm, allowing time to dry the plate in between runs, took only 2–2$^1/_2$ h (Fig. 1).

Visualisation of steroids on chromatoplates

Oe and Andien and their acetates gave distinctive colours with PMA, PTA, RA and the Allen reagent. The colours developed with PMA, RA and the Allen reagent by heating the plates at 110° for about 4 min, but only Oe and Andien and their acetates gave colours with PTA in this time. The other steroids and particularly the acetates of An (α), Ae and An (β) required about 8 min heating at 110°. On standing in daylight for about 20 min., all the colours with PTA had faded to mauve except those of Oe and Oe acetate which turned red. Iodine in light petroleum was found useful since

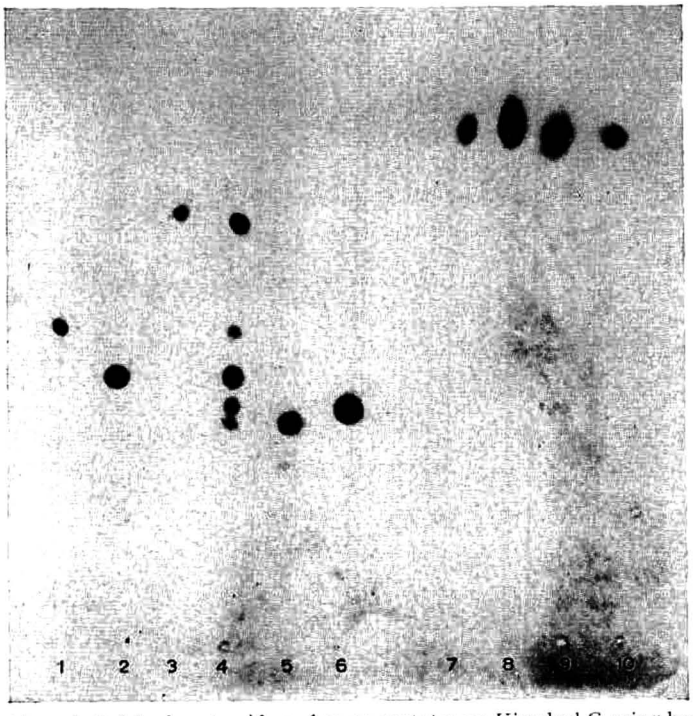


Fig. 1. Separation of 16-dehydro-steroids and some acetates on Kieselgel G using benzene–methyl ethyl ketone (9:1 v/v) as mobile phase. The plate was run three times allowing time for the solvent to evaporate between each run. 1 = 5α-androst-16-en-3α-ol; 2 and 7 = 5β-androst-16-en-3α-ol and acetate; 3 and 8 = oestra-1,3,5(10),16-tetraen-3-ol and acetate; 5 and 9 = 5α-androst-16-en-3β-ol and acetate; 6 and 10 = androsta-5,16-dien-3β-ol and acetate; 4 = mixture of 1, 2, 3, 5 and 6.

all the compounds gave yellow spots in the cold. The reaction appeared to be reversible since the spots faded completely after about 30 min. Most of the compounds gave specific colours with the uranyl nitrate reagent; 6–7 min heating at 110° was required (Table III).

Use of silicic acid-impregnated paper

The success with which phosphatides have been separated on silicic acid-impregnated paper in past years[14] suggested the possibility of applying this technique to the separation of C_{19}-16-dehydro-steroids and their acetates. The results obtained, summarized in Table III, were no better than those using the thin layer technique. Prolonged running of some systems overnight using the ascending technique greatly enhanced the separation of An (α) and Ac but An (β) and Andien still moved as one spot (Table IV).

The resolution of these steroids on fully acetylated paper or in systems using phenylcellosolve, kerosene and liquid paraffin as stationary phase, was disappointing. An (α) could be separated fairly easily from Ac, An (β) and Andien, but there was little difference, however, between the mobility of the androstenol analogues. Alteration of the mobile phase, the amount of stationary phase in the paper and overrunning of chromatograms produced no better results.

TABLE III

APPROXIMATE R_F VALUES OF SOME 16-DEHYDRO-STEROIDS AND THEIR ACETATES ON SILICIC ACID-IMPREGNATED PAPER

*Compound**	*Solvents***											*Colours with visualising reagents****			
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*PMA**	*PTA**	*RA**	*Allen*[20]
An (α)	0.17	0.47	0.52	0.38	0.33	0.61	0.59	0.63	0.42	0.30	0.91	bg	pk	m	m
Ae	0.10	0.36	0.42	0.30	0.25	0.53	0.47	0.55	0.34	0.21	0.88	bg	pk	m	m
An (β)	0.08	0.29	0.34	0.26	0.18	0.46	0.41	0.48	0.28	0.17	0.84	bg	pk	m	y
Andien	0.09	0.31	0.37	0.28	0.20	0.47	0.43	0.50	0.31	0.19	0.85	p	ro	b	m
Oe	0.24	0.64	0.69	0.51	0.46	0.77	0.71	0 74	0.55	0.40	0.95	m	o	or	ro
An (α) acetate	0.55	0.84	0.86	0.95	0.68	1.0	0.91	0.85	0.72	0.60	1.0	bg	pk	m	m
Ae acetate	0.61	0.85	0.87	0.96	0.69	1.0	0.92	0.87	0.72	0.64	1.0	bg	pk	m	m
An (β) acetate	0.60	0.85	0.88	0.95	0.70	1.0	0.92	0.87	0.71	0.65	1.0	bg	pk	m	y
Andien acetate	0.60	0.86	0.87	0.94	0.69	1.0	0.92	0.87	0.70	0.64	1.0	p	ro	b	m
Oe acetate	0.60	0.86	0.88	0.96	0.71	1.0	0.94	0.90	0.72	0.65	1.0	m	o	r	p

* For abbreviations see Experimental Section.

** Solvents; abbreviations: 1 = light petroleum (b.p. 83–97°)–benzene, 1:1 (v/v); 2 = benzene; 3 = benzene–ether, 99:1 (v/v); 4 = toluene–ether, 99:1 (v/v); 5 = toluene–ethyl acetate, 99:1 (v/v); 6 = ethylene chloride; 7 = benzene–methylene chloride, 50:50 (v/v); 8 = benzene–ethylene chloride, 50:50 (v/v); 9 = benzene–methyl ethyl ketone, 99:1 (v/v); 10 = toluene; 11 = methylene chloride.

*** Colours; abbreviations: b = blue; bg = bluish green; m = mauve; o = orange; r = red; or = orange red; ro = rose; p = purple; pk = pink y = yellow

TABLE IV

MOBILITY IN CM/HOUR OF SOME 16-DEHYDRO-STEROIDS RUN ON SILICIC ACID IMPREGNATED PAPER FOR 18 HOURS*

*Compound***	*Solvent*		
	Light petrol–benzene 1 : 1 (v/v)	*Benzene*	*Benzene–ether 995 : 5 (v/v)*
An (α)	0.54	1.0	—
Ae	0.40	0.75	0.86
An (β)	0.26	0.62	0.70
Andien	0.26	0.62	0.70

* Chromatography was performed using the ascending technique. A pad of filter paper was clipped to the upper end of the chromatogram to absorb solvent.
** Abbreviations are given in Experimental Section.

DISCUSSION

The order of mobilities of the 16-dehydro-steroids (Oe > An (α) > Ae > Andien > An (β)) was constant in all the solvents used both on Kieselgel G and on silicic acid-impregnated paper. Resolutions were slightly better on the former due presumably to the compactness of the spots obtained. For practical purposes it was possible to separate Oe, An (α) and Ae from each other and from An (β) and Andien on thin layer plates if the overrunning technique was used. The difference in mobility between An (β) and Andien was too small, however, to allow anything better than a separation for the purposes of identification although Andien could be distinguished in a mixture by virtue of the specific colours given with PMA and RA.

The iodine reagent was useful in that all the compounds tested gave yellow spots in the cold. Moreover, the reaction was reversible so that a whole plate, consisting of marker lanes and unknown lanes, could be sprayed with no danger of some iodine spots remaining. It should be pointed out, however, that with iodine in vapour form, 10–20 μg quantities of all the compounds gave intense, yellow-brown spots which faded only slowly and incompletely so that if these were rechromatographed the yellow-brown, presumed iodo-derivatives remained at the origin (BROOKSBANK, unpublished). This could lead to serious errors in quantitative measurements.

The difficulty with which this series of 16-dehydro-steroids could be separated has confirmed conclusions previously made using gas chromatography[4] but improved resolution might be obtained using the trimethyl silyl ethers[19]. On theoretical grounds better separations should also be possible if a capillary column equivalent to 45,000 theoretical plates is employed[4]. Another approach to this problem makes use of the fact that when dehydro*epi*androsterone and *epi*androsterone, another pair of 3β-hydroxy-steroids which are difficult to separate[20] are oxidised, the corresponding 3,17-di-ketones can be resolved easily. Accordingly, experiments are being pursued to determine whether the 3-ketones corresponding to An (β) and Andien can be separated.

ACKNOWLEDGEMENTS

I am grateful to Prof. G. A. D. HASLEWOOD for continued interest in this work and to Dr. B. W. L. BROOKSBANK for constructive criticism and for permission to quote unpublished results.

SUMMARY

The chromatography of a closely related series of 16-dehydro-steroids and their acetates on thin layer plates and silicic acid-impregnated paper is described. Fairly rapid and easy separations of most of the compounds studied can be achieved although the separation of androst-16-en-3β-ol and androsta-5,16-dien-3β-ol is only obtained by an overrunning technique. Silicic acid-impregnated paper appears to be free from disadvantages associated with papers impregnated with other stationary phases such as phenylcellosolve, liquid paraffin and kerosene. The use of a number of colour reagents is described, including phosphomolybdic acid, phosphotungstic acid, resorcylaldehyde–sulphuric acid, the Allen reagent and uranyl nitrate in sulphuric acid. Some of the steroids give specific colours which may be useful in their identification.

NOTE ADDED IN PROOF

Since this paper was submitted excellent separations of the trimethyl silyl ethers of An (α), Ae, An (β) and Andien have been obtained by gas chromatography on a QF 1 column.

REFERENCES

1 B. W. L. BROOKSBANK AND G. A. D. HASLEWOOD, *Biochem. J.*, 80 (1961) 488.
2 D. B. GOWER AND G. A. D. HASLEWOOD, *J. Endocrinol.*, 23 (1961) 253.
3 D. B. GOWER, *J. Endocrinol.*, 26 (1963) 173.
4 R. W. R. BAKER AND D. B. GOWER, *Nature*, 192 (1961) 1074.
5 B. W. L. BROOKSBANK, *J. Endocrinol.*, 24 (1962) 435.
6 M. BARBIER, H. JÄGER, H. TOBIAS AND E. WYSS, *Helv. Chim. Acta*, 42 (1959) 2440.
7 M. J. D. VAN DAM, G. J. DE KLEUVER AND J. G. DE HEUS, *J. Chromatog.*, 4 (1960) 26.
8 M. VAN DAM, *Bull. Soc. Chim. Belg.*, 70 (1961) 122.
9 R. NEHER AND A. WETTSTEIN, *Helv. Chim. Acta*, 35 (1952) 276.
10 B. L. RUBIN, R. I. DORFMAN AND G. PINCUS, *J. Biol. Chem.*, 203 (1953) 629.
11 R. P. MARTIN, *Biochim. Biophys. Acta*, 25 (1957) 408.
12 E. KODICEK AND D. R. ASHBY, *Biochem. J.*, 57 (1954) xii.
13 F. J. RITTER AND J. HARTEL, *J. Chromatog.*, 1 (1958) 461.
14 C. H. LEA, D. N. RHODES AND R. D. STOLL, *Biochem. J.*, 60 (1955) 353.
15 E. STAHL, *Chemiker-Ztg.*, 82 (1958) 323; *Parfuem. Kosmetik*, 39 (1958) 564.
16 D. KRITCHEVSKY AND M. R. KIRK, *Arch. Biochem. Biophys.*, 35 (1952) 346.
17 I. E. BUSH, *Nature*, 166 (1950) 445.
18 W. M. ALLEN, S. J. HAYWARD AND A. PINTO, *J. Clin. Endocrinol. Metab.*, 10 (1950) 54.
19 M. A. KIRSCHNER AND M. B. LIPSETT, *J. Clin. Endocrinol. Metab.*, 23 (1963) 255.
20 S. H. WEINMANN, O. CREPY, E. E. BAULIEU, J. GUY AND M. F. JAYLE, *Bull. Soc. Chim. Biol.*, 39 (1957) 463.

IDENTIFIZIERUNG VON GLYCIDYLÄTHERN

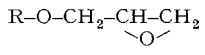
TRENNUNG UND IDENTIFIZIERUNG VON α-ALKYL(ARYL)-ÄTHERN DES GLYCERINS MIT HILFE DER PAPIERCHROMATOGRAPHIE

V. ULBRICH UND V. DLASK

Forschungsinstitut für Synthetische Harze und Lacke, Pardubice (Tschechoslowakei)

(Eingegangen den 9. September 1963)

Glycidyläther sind organische Verbindungen der allgemeinen Formel:



Sie enthalten im Molekül eine cyclische Äthergruppe, deren Sauerstoffatom mit den beiden benachbarten Kohlenstoffatomen einen dreigliedrigen Ring bildet

Als Folge der durch den dreigliedrigen Ring bedingten starken Spannung weisen die Glycidyläther eine hohe Reaktivität auf und werden fast von allen nucleophilen Stoffen angegriffen. Die Öffnung des Rings wird unter gleichzeitiger Bildung von Additionsverbindungen durch Halogensäuren, Sulfosäuren, saure Sulfite, Thiosulfate, Carbonsäuren, Cyanwasserstoffsäure, Wasser, Amine usw. bewirkt.

Die Glycidyläther wurden trotz ihrer hohen Reaktivität vom Standpunkt ihrer möglichen Identifizierung bisher nicht systematisch studiert. Die Ursache hierfür ist einerseits auf deren Reaktivität, andererseits auf die Möglichkeit der Bildung von isomeren Derivaten bei der Öffnung des Epoxydrings zurückzuführen. Von der Molekülstruktur des Glycidyläthers können theoretisch zwei Identifizierungsarten abgeleitet werden. Die beiden Arten beruhen in der Spaltung der ätherischen Bindung. Durch Erwärmung mit der Jodwasserstoffsäure nach Zeisel kann die Bindung R–O gespalten und das entstandene Alkyljodid nach erfolgter Isolierung aus dem Reaktionsmedium in ein kristallinisches Derivat überführt werden.

Bei den niederen Gliedern der homologen Reihe von Glycidyläthern kann die Isolierung des entsprechenden Alkyljodids durch Destillierung im Strom eines Inertgases erfolgen. Höhere Alkyljodide können jedoch infolge ihrer hohen Siedepunkte quantitativ nicht mehr destilliert und in der Vorlage mit einer geeigneten Absorptionslösung aufgefangen werden. Es wäre dann notwendig, Alkyljodid aus einem verhältnismässig komplizierten Reaktionsmedium auf chemischen Wege zu isolieren, was mit erheblichen Schwierigkeiten verbunden wäre und verschiedene Nachbehandlungen erfordern würde.

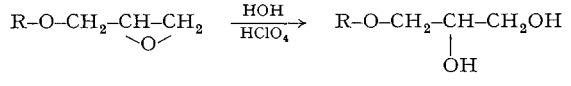
Aus diesem Grunde haben wir von der zweiten möglichen Identifizierungsart

Gebrauch gemacht. Dies setzt die Öffnung des Epoxydrings mit Hilfe des Reagens voraus, das direkt ein kristallinisches Derivat oder wenigstens ein Produkt einer einheitlichen und definierbaren Zusammensetzung, aus welchem durch sekundäre Reaktion mit einem geeigneten Reagens ein kristallinisches Derivat dargestellt werden kann. Durch Hydratation von Monoglycidyläthern haben wir die funktionelle Epoxydgruppe in primäre und sekundäre Hydroxylgruppen des Glycerins überführt, was die Identifizierung durch klassische Identifizierungsverfahren als auch durch moderne analytische Methoden, wie z.B. durch Papierchromatographie ermöglichte.

Die Glycidyläther kann man wegen ihrer Flüchtigkeit und der begrenzten Detektion nicht als solche durch Papierchromatographie anwenden. Sie sind in geeignete nichtflüchtige Derivate zu überführen, deren Eigenschaften gleichzeitig für die empfindliche Detektion auf dem Chromatogramm verwendet werden könnten.

Bisher wurden Glycidyläther als Reaktionsprodukte mit Phenolsulfonsäuren oder mit Pikrinsäure[1] chromatographisch untersucht. Der Nachteil dieser Methoden ist die Darstellung der zugehörigen Stoffe und die Möglichkeit des Verlaufes von Nebenreaktionen. SCHÄFER[1] führt an, dass durch Reaktion von Sulfosalicylsäure und Pikrinsäure mit Glycidyläther Stellungsisomere erhalten werden, die auf dem Chromatogramm zwei Flecke bilden. Diese Tatsache ist verständlich unerwünscht, insbesondere bei der Identifizierung einer Mehrkomponentenmischung.

In unserer Arbeit haben wir für die chromatographische Trennung von Glycidyläthern deren Derivate, die α-Alkyl(Aryl)-Äther des Glycerins verwendet. Durch katalytische Hydratation von Glycidyläthern haben wir unter gleichzeitiger Öffnung



des Epoxydrings die zugehörigen α-Glycerinäther gewonnen, und somit die Möglichkeit der Bildung von Stellungsisomeren vermieden. Die Glycerinäther weisen eine niedrigere Flüchtigkeit auf und haben geeignete Eigenschaften für die Anwendung der chromatographischen Trennungsmethodik.

Die Chromatographie von Glykolen, Glycerin und deren Derivate wurde von zahlreichen Autoren beschrieben. BERGNER UND SPERLICH[2] haben für die Trennung dieser Stoffklasse nicht vorbehandelte Papiere und im System Chloroform–Äthanol, oder Äther mit Wasser gesättigt, eine gute Trennung von Äthylenglykol, Propylenglykolen und Butylenglykolen erzielt. HOUGH[3] empfiehlt für die Trennung von ähnlichen Verbindungen Butanol, Äthanol und Wasser bzw. Benzol, Butanol und Pyridin enthaltendes System. Eine Reihe von weiteren Arbeiten nimmt von der Möglichkeit der Esterifizierungs- oder Ätherifizierungsreaktionen Gebrauch und führt die genannten Verbindungen in Xanthate[4], 3,6-Dinitrophthalate[5] oder 3,5-Dinitrobenzoate[6] über.

EXPERIMENTELLER TEIL

Darstellung der Stoffe für die Chromatographie

Die präparative Darstellung von α-Alkyl(Aryl)-Glycidyläthern und deren Überführung durch katalytische Hydratation in α-Alkyl(Aryl)-Äther des Glycerins sowie die physikalischen Eigenschaften beider Reihen wurden auf einer anderen Stelle[7] be-

schrieben. Für die eigentliche chromatographische Trennung wurden 5 %ige Lösungen von α-Alkyl(Aryl)-Äthern des Glycerins in Äthanol verwendet. Im allgemeinen kann man folgendes Verfahren empfehlen:

Zu 2 mMol Glycidyläther werden 15 ml Wasser und 1 Tropfen Perchlorsäure (75 %ige) zugesetzt. Das Gemisch wird auf siedendem Wasserbad 2 Stunden lang erwärmt (die Probe auf die Anwesenheit von Epoxydgruppen negativ), mit verdünnter NaOH-Lösung neutralisiert und mit 5 ml Äther ausgeäthert. Die ätherische Lösung wird eingedampft und direkt auf das Chromatogramm aufgetragen. Die Isolierung von Glycerinäthern kann gleichfalls unter Wasserabdunstung unter vermindertem Druck (10 mm Hg) und durch Extraktion des Destillierrückstands in 2 ml Äthanol erfolgen. Die äthanolische Lösung von Glycerinäthern wird zum Auftragen auf das Chromatogramm verwendet.

Chromatographie

Das Papier Whatman Nr. 3 wurde mit 15 %iger äthanolischer Formamidlösung imprägniert und bei Raumtemperatur 10–15 Min. lang in der Luft eingehängt. Auf das imprägnierte Papier wurden 7 cm vom oberen Rand und 3 cm voneinander mit der Mikropipette Lösungen von Glycerinäthern in Äthanol in der Menge von 250–1000 μg aufgetragen. Es wurde absteigend auf übliche Weise chromatographiert. Als Laufmittel wurde das Gemisch Benzol–Äthanol (40:5) verwendet. Die Lösungsmittel legen eine Strecke von 33–35 cm in 120–150 Min. zurück. Hierauf wurde das Chromatogramm herausgenommen und in der Luft eingehängt, damit das Lösungsmittel sich verflüchtigt.

Die Detektion von Glycidyläthern auf dem Chromatogramm

Nach Verflüchtigen des Lösungsmittels aus dem Papier wurde das Chromatogramm mit einer gesättigten Lösung von Kaliumperjodat besprüht und nach 7 Min. Einwirkung wurde die Lösung von 0.1 *M* Benzidin in 50 %iger Methanol mit Aceton und 0.2 *N* HCl (10:2:1) verwendet. Die Glycerinäther mit einem freien *o*-Diol-Aufbau erschienen als weisse Flecke auf blauem Hintergrund. Die Lösung von Benzidin in Äthanol wurde jeden Tag frisch vorbereitet. Mit dem genannten Verfahren wurden Chromatogramme mit scharfen und kreisförmigen Flecken gewonnen.

Ergebnisse und Diskussion

Zur chromatographischen Trennung von α-Alkyl(Aryl)-Äthern des Glycerins haben sich die mit Formamid und Dimethylformamid imprägnierten Papiere bewährt, da in diesen stationären Phasen die polaren Glycerinäther sehr gut löslich sind. Somit wird es möglich genügend grosse Mengen der Glycerinäther (250–1000 μg) ohne Bildung von Streifen zu chromatographieren, was in Verbindung mit einem empfindlichen Nachweisverfahren den Nachweis einer kleinen Menge eines Glycerinäthers neben dem Überschuss eines anderen Glycerinäthers gestattet. Wir haben auf diese Weise z.B. noch 0.5 % α-*n*-Amyläther des Glycerins in α-*n*-Nonyläther des Glycerins nachgewiesen.

Als durchfliessende Phasen haben wir das Gemisch von Benzol–Äthanol bezw. von Hexan–Pyridin–Chloroform verwendet. Diese Lösungsmittel wandern sehr schnell durch das Papier, sodass die Laufzeit des Chromatogramms 120–150 Min. beträgt. Im Gemisch von Hexan–Pyridin–Chloroform (40:5:1) bei Verwendung der

TABELLE I

R_F UND R_M-WERTE VON α-ALKYL(ARYL)-ÄTHERN DES GLYCERINS

Papier: Whatman Nr. 3. Imprägnierung: 15%iges äthanolisches Formamid. System: Benzol–Äthanol (40:5). Laufzeit: 120 Min. Detektion: KJO_4, Benzidin. Temperatur: 18–20°. Länge des Chromatogramms: 33 cm.

Glycerinäther (GÄ)	R_F	R_M
Methyl-GÄ	0.02	1.69
Äthyl-GÄ	0.04	1.38
Isopropyl-GÄ	0.09	1.00
n-Propyl-GÄ	0.08	1.06
Isobutyl-GÄ	0.15	0.75
n-Butyl-GÄ	0.16	0.72
n-Amyl-GÄ	0.28	0.41
n-Hexyl-GÄ	0.47	0.05
n-Heptyl-GÄ	0.64	—0.25
n-Octyl-GÄ	0.77	—0.52
n-Nonyl-GÄ	0.85	—0.74
Allyl-GÄ	0.06	1.20
Phenyl-GÄ	0.13	0.83
Tolyl-GÄ	0.22	0.55
p-tert.-Butylphenyl-GÄ	0.63	0.24
Cyclohexyl-GÄ	0.26	0.45
Benzyl-GÄ	0.16	0.72

1%igen Lösung von Dimethylformamid als stationäre Phase weisen α-Alkyläther des Glycerins die niedrigsten R_F-Werte auf. Mit der zunehmenden Polarität des Systems erhöhen sich auch die R_F-Werte. Diese Tatsache kann für eine gute Trennung ausgenützt werden. Durch eine geeignete Kombination der angeführten bezw. anderer Systeme können beliebige R_F-Werte erzielt und somit von Fall zu Fall die erforderlichen Trennungen durchgeführt werden.

In Tabelle I und II sind R_F-Werte jener Systeme angeführt, die sich bestens bewährt haben. Zur Detektion von α-Alkyl(Aryl)-Äthern haben wir empfindliche,

TABELLE II

R_F-WERTE VON GLYCERIN-ALKYLÄTHERN

Papier: Whatman Nr. 3. Imprägnierung: I_1 = 10%ige, I_2 = 5%ige, I_3 = 1%ige methanolische Lösung von Dimethylformamid. System: S_1 = Hexan–Pyridin–Chloroform (40:7:3). S_2 = Hexan–Pyridin–Chloroform (40:10:5). S_3 = Hexan–Pyridin–Chloroform (40:5:1). Laufzeit: 120 Min. Detektion: KJO_4, Benzidin. Temperatur: 18–20°. Länge des Chromatograms: 33 cm.

Glycerinäther	S_1			S_2			S_3		
	I_1	I_2	I_3	I_1	I_2	I_3	I_1	I_2	I_3
Methyl-GÄ	0.02	0.05	0.01	0.07	0.09	0.11	0.01	0.01	0.00
Äthyl-GÄ	0.04	0.10	0.02	0.10	0.13	0.17	0.03	0.04	0.00
n-Propyl-GÄ	0.08	0.17	0.10	0.17	0.29	0.28	0.10	0.10	0.01
n-Butyl-GÄ	0.14	0.20	0.20	0.27	0.43	0.39	0.17	0.18	0.06
n-Amyl-GÄ	0.24	0.43	0.32	0.43	0.57	0.49	0.26	0.29	0.16
n-Hexyl-GÄ	0.37	0.57	0.42	0.54	0.64	0.58	0.32	0.37	0.28
n-Heptyl-GÄ	0.55	0.66	0.54	0.67	0.73	0.66	0.42	0.47	0.46
n-Octyl-GÄ	0.66	0.76	0.65	0.78	0.79	0.72	0.50	0.54	0.60
n-Nonyl-GÄ	0.77	0.81	0.72	0.86	0.85	0.79	0.60	0.60	0.66

üblicherweise für die Detektion von Zuckern[8] verwendeten oxydativreduzierende Reaktionen benützt. Durch Besprühen des Chromatogramms mit der Lösung von Kaliumperjodat und mit der methanolischen Benzidinlösung erschienen die zugehörigen Glycerinäther als weise Flecke am blauen Hintergrund. Eine optimale Trennung von α-Alkyl(Aryl)-Äthern des Glycerins haben wir unter Verwendung von Benzol und Äthanol (Figs. 1 und 2) auf den mit Formamid imprägnierten Papieren gewonnen. Auch das System Hexan–Pyridin–Chloroform und mit Dimethylformamid behandeltes Papier gewähren eine gute Trennung der einzelnen Glyzerinäther.

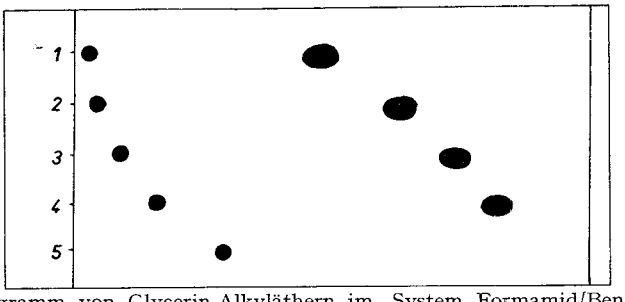


Fig. 1. Chromatogramm von Glycerin-Alkyläthern im System Formamid/Benzol–Äthanol. (1) Methyl- und Hexyläther des Glycerins; (2) Äthyl- und Heptyläther des Glycerins; (3) Propyl- und Octyläther des Glycerins; (4) Butyl- und Nonyläther des Glycerins; (5) Amyläther des Glycerins.

Bezüglich der Beziehung zwischen den R_F-Werten und der Struktur der chromatographierten Stoffe, verursacht das Anwachsen der Kohlenstoffkette von Alkylen in den Glycerinäthern die Erhöhung der R_F-Werte. Die Regelmässigkeit in der homologen Reihe von Glycerinäthern kann man in den R_M-Werten ausdrücken, die wie folgt gekennzeichnet[9] sind:

$$R_M = \log\left(\frac{1}{R_F} - 1\right)$$

Die Abhängigkeit dieser Werte im R_F Bereich 0.1–0.75 von der Anzahl der Kohlenstoffatome der chromatographierten Stoffe ist geradlinig (Fig. 4). Die Zunahme des R_M-Wertes für eine $-CH_2$-Gruppe beträgt im Durchschnitt 0.33 für die Substitution in der aliphatischen Kette. Ähnlich wie bei den anderen homologen

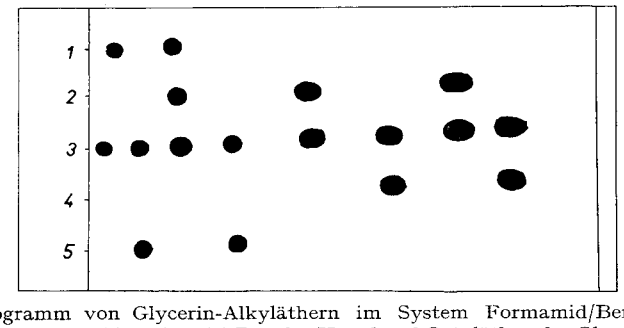


Fig. 2. Chromatogramm von Glycerin-Alkyläthern im System Formamid/Benzol–Äthanol. (1) Äthyl- und Butyläther des Glycerins; (2) Butyl-, Hexyl und Octyläther des Glycerins; (3) Methyl-, Propyl-, Butyl-, Amyl-, Hexyl-, Heptyl-, Octyl- und Nonyläther des Glycerins; (4) Heptyl- und Nonyläther des Glycerins; (5) Propyl- und Amyläther des Glycerins.

Reihen sind die R_F-Werte bei den Isomeren sehr nahe, sodass sie praktisch nicht getrennt werden. Die Anwesenheit von wenig polaren Methylgruppen am Benzolring macht sich bedeutend bemerkbar und ermöglicht auf diese Weise die chromatographische Trennung von Phenyl- und Tolyläther vom *p-tert.*-Butylphenyläther des Glycerins (Fig. 3). Zu den angeführten Beziehungen ist es jedoch zu bemerken, dass

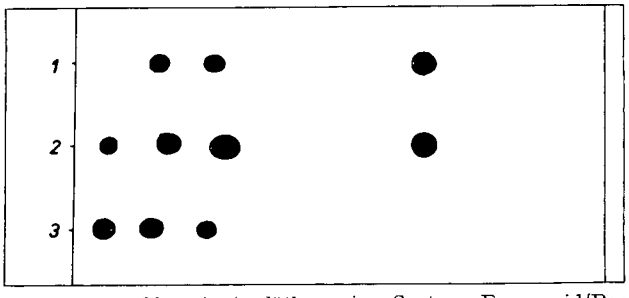


Fig. 3. Chromatogramm von Glycerin-Aryläthern im System Formamid/Benzol–Äthanol. (1) Phenyl-, Tolyl- und *p-tert.*-Butylphenyläther des Glycerins; (2) Allyl-, Benzyl-, Cyclohexyl- und *p-tert.*-Butylphenyläther des Glycerins; (3) Allyl-, Phenyl- und Tolyläther des Glycerins.

die R_F-Werte bei der Chromatographie auf imprägnierten Papieren eine höhere Streuung aufweisen, weil sie von der Imprägnierungsart, der Zeit der Papiertrocknung, der Luftfeuchtigkeit und von der Sättigung der Kammer mit den beiden Phasen[10,11] abhängen. Es ist daher nötig die angeführten Faktoren womöglich konstant zu halten und bei der Identifizierung des unbekannten Glycidyläthers die Referenzstoffe gleichzeitig zu chromatographieren.

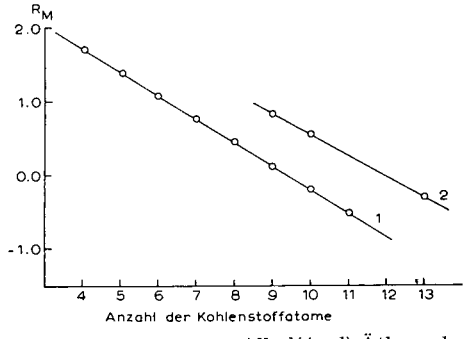


Fig. 4. R_M-Werte der homologen Reihen von α-Alkyl(Aryl)-Äthern des Glycerins. (1) Alkyläthern des Glycerins (Formamid/Benzol–Äthanol). (2) Aryläthern des Glycerins (Formamid/Benzol–Äthanol).

ZUSAMMENFASSUNG

Es wurde eine Methode zur Trennung und Identifizierung von Glycidyläthern als α-Alkyl(Aryl)-Äthern des Glycerins mittels Chromatographie auf mit Formamid und Dimethylformamid imprägniertem Papier beschrieben.

Durch Reaktion von Epichlorhydrin mit zugehörigem Alkohol (Phenol) wurde eine Reihe von Alkyl- und Arylglycidyläthern dargestellt. Die reaktive Epoxyd-

funktion von Glycidyläthern wurde durch Hydratation unter katalytischer Einwirkung von Perchlorsäure in primäre und sekundäre Hydroxylfuktionen von Alkyl(Aryl)-Äthern des Glycerins überführt. Die letztgenannten Stoffe wurden für die Trennung und Identifizierung durch Papierchromatographie verwendet. Auf den im System Benzol–Äthanol und Hexan–Pyridin–Chloroform mit Formamid und Dimethylformamid behandelten Papieren wurde eine sehr gute Trennung der homologen Reihe von Glycerinäthern erreicht.

Die beschriebene Methodik kann für die Identifizierung verschiedener Glycidyläther, die als reaktive Lösungsmittel eine weitgehende Bedeutung für die Synthese von Epoxydharzen haben, verwendet werden.

SUMMARY

A method is described for the separation and identification of glycide ethers, in which these ethers are converted into α-alkyl(aryl) ethers of glycerol and then subjected to chromatography on paper impregnated with formamide or dimethylformamide.

A series of alkyl and aryl glycide ethers was prepared by allowing epichlorohydrin to react with the appropriate alcohol or phenol. The reactive epoxy group of the glycide ethers was hydrated, using perchloric acid as catalyst, to give the primary and secondary alcohol functions, so that alkyl(aryl) ethers of glycerol were obtained. These ethers were then chromatographed on paper. Using paper treated with formamide or dimethylformamide and the solvent systems benzene–ethanol or hexane–pyridine–chloroform, very good separation of the homologous glycerol ethers was obtained.

This method can be used to identify various glycide ethers, which are of importance as reactive solvents in the synthesis of epoxy resins.

LITERATUR

[1] W. SCHÄFER, W. NUCK UND H. JAHN, *J. Prakt. Chem.*, 11 (1960) 1, 11.
[2] K. G. BERGNER UND H. SPERLICH, *Z. Lebensm. Untersuch.-Forsch.*, 97 (1953) 253.
[3] L. HOUGH, *Nature*, 165 (1950) 400.
[4] T. KARIYONE, S. SHIMIZU UND Y. HASHIMOTO, *Nature*, 170 (1952) 422.
[5] T. MOMOSE UND A. YAMADA, *J. Pharm. Soc. Japan*, 71 (1951) 980; *C. A.*, 46 (1952) 1921.
[6] J. BORECKÝ UND J. GASPARIČ, *Collection Czech. Chem. Commun.*, 25 (1960) 1287.
[7] V. ULBRICH, J. MAKEŠ UND M. JUREČEK, *Collection Czech. Chem. Commun.*, im Druck.
[8] I. M. HAIS UND K. MACEK, *Papierchromatographie*, ČSAV, Prag, 1959.
[9] E. C. BATE-SMITH UND R. G. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.
[10] J. GASPARIČ UND M. VEČEŘÁ, *Chem. Listy*, 51 (1957) 287.
[11] E. SUNDT UND M. WINTER, *Anal. Chem.*, 29 (1957) 851; 30 (1958) 1620.

PAPIERCHROMATOGRAPHIE ÄTHERISCHER ÖLE

I. EIN NEUES VERFAHREN ZUR DIREKTEN PAPIERCHROMATOGRAPHISCHEN TRENNUNG ÄTHERISCHER ÖLE AUF IMPRÄGNIERTEM PAPIER

G. RICHTER* UND P. MUSCHOLL

München (Deutschland)

(Eingegangen den 25. September 1963)

In einer Kurzmitteilung[1] berichteten wir über ein neues papierchromatographisches Trennverfahren für unbehandelte ätherische Öle und deren Bestandteile, das auch eine direkte photometrische Bestimmung ohne Elution ermöglicht. Die vorliegende Arbeit bringt eine ausführliche Beschreibung dieses Verfahrens**.

BESCHREIBUNG DES VERFAHRENS

Adsorbierendes Chromatogrammpapier

Da die Trennung ätherischer Öle auf unbehandeltem Chromatographierpapier bisher praktisch undurchführbar war, versuchten wir ein geeignetes Papier mit hydrophoben und adsorptiven Eigenschaften herzustellen, das sich für solche Trennungen eignete. Wir wählten zunächst Phenolkunstharze verschiedener Polymerisationsstufen. Mit den gebräuchlichen Fliessmitteln war jedoch immer noch eine mehr oder weniger starke Löslichkeit der eingelagerten Phenoplaste zu beobachten. Nach zahlreichen Experimenten bewährte sich dagegen in Fortführung früherer Arbeiten[2,3] eine direkte Imprägnation des Papieres mit Paraformaldehyd. Als Ausgangsmaterial verwendeten wir nach Testung anderer Papiersorten Ederol 208 der Fa. J. C. Binzer, Hatzfeld/Eder. Durch die Imprägnierung erhielt das Papier ausser einem beachtlichen Adsorptionsvermögen hydrophobe und ionenaustauschende Eigenschaften. Die Reissfestigkeit im nassen Zustand war gegenüber den unbehandelten Papieren erhöht.

Vergleiche ergaben, dass das imprägnierte Papier bei Verwendung geeigneter Fliessmittel etwa die gleichen Trennleistungen aufweist wie Kieselgel G-Dünnschichtplatten[4–8]. Die Trennung der Terpenalkohole Geraniol, Linalool und Citronellol gelingt z.B. jedoch auf unserem Papier*** wesentlich besser (Fig. 1).

Die Entwicklungsdauer reicht an die kurzen Zeiten der Dünnschichtchromatographie heran. Für Routineuntersuchungen lässt sich auch die Rundfilterchromatographie heranziehen (Fig. 2).

* Dr. Günther Richter, 8 München 15, Platenstr. 6, Deutschland.

** Die densitometrischen Bestimmungen werden in einer weiteren Arbeit beschrieben.

*** Das Papier wird nach unseren Angaben von der Fa. J. C. Binzer, Hatzfeld/Eder (Deutschland), unter der Bezeichnung Ederol 208/P hergestellt.

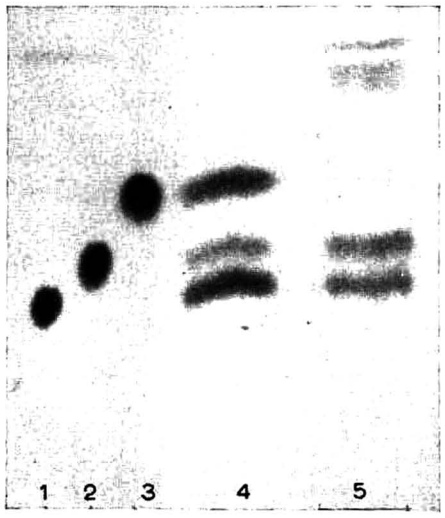


Fig. 1. Papierchromatogramm eines ägyptischen Geraniumöles (Trennung von Geraniol, Citronellol und Linalool). Methode: aufsteigend mit Fliessmittel 5 auf mit Paraformaldehyd imprägniertem Papier; Laufzeit: 90 Min.; Anfärbung: Osmiumtetroxid. 1 = Geraniol; 2 = Citronellol; 3 = Linalool; 4 = Gemisch aus Geraniol, Citronellol und Linalool; 5 = ägyptisches Geraniumöl.

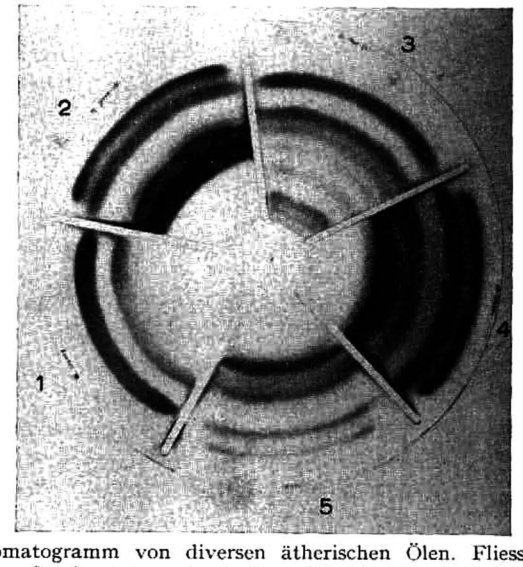


Fig. 2. Rundfilterchromatogramm von diversen ätherischen Ölen. Fliessmittel: 1; Laufzeit: 20 Min.; Anfärbung: Osmiumtetroxyd, Antimon(V)-chlorid und 2,4-Dinitrophenylhydrazin nacheinander (Front am Ende der gestanzten Löcher). 1 = Lavendelöl Mt. Blanc; 2 = afrikanisches Geraniumöl; 3 = Neroliöl; 4 = javanisches Citronellöl; 5 = Melissenöl des Handels.

Chromatographiearten

Für die meisten Aufgaben hat sich die aufsteigende Methode bewährt (Fig. 3). Dabei genügt eine Laufstrecke von 15–18 cm. Eine gute Absättigung der Kammer verkürzt die Laufzeiten und verbessert die Trennungen. Sie wird erreicht durch Auskleiden der Kammerwände mit Filtrierpapier, das mit der mobilen Phase getränkt ist.

Für besonders schwierige Trennaufgaben ist die absteigende Chromatographie heranzuziehen, so z.B. zur sauberen Unterscheidung der Substanzpaare Geraniol–Farnesol und Anisaldehyd–Zimtaldehyd (Fig. 4).

Bei Serienanalysen ist die Rundfilterchromatographie die Methode der Wahl. Als Kammern können dabei Exsikkatoren oder Petrischalen[9] dienen. Da die Petrischalen nicht abdichtbar sind, muss ein möglichst grosses Uhrglas als Behälter für das Fliessmittel verwendet werden, damit die Kammeratmosphäre weitgehend gesättigt wird. Der Docht soll nicht dicker als etwa 2 mm sein, um gleichmässig schmale Zonen zu erhalten. Packschnur von der Dicke einer Präpariernadel ist für kleine Rundfilter (14.5 cm Durchmesser) gut geeignet. Die Laufzeit beträgt 20–45 Min. je nach Durchmesser des Rundchromatogramms, mit Fliessmittel 1. (Siehe Tabelle II.)

Fliessmittel

Die ausgearbeiteten Gemische sind einphasig. Für die Chromatographie der meisten ätherischen Öle eignet sich eine Mischung von *n*-Hexan–*n*-Heptan–Eisessig. Dieses System lässt sich durch Zusatz von Toluol (a) oder mehr *n*-Heptan (b) so verändern, dass die R_F-Werte beliebig erhöht (a) oder gesenkt (b) werden können. Speziell für Lavendel-, Lavandin- und Spiköle hat sich Cyclohexan–Äthylacetat oder auch Trichloräthylen bewährt. Die Trennung der Alkohole Geraniol, Linalool und Citronellol gelingt mit *n*-Heptan–Methylisopropylketon-Gemisch (siehe Tabelle II).

Anfärbeverfahren

Versuche, die bei der Dünnschichtchromatographie von ätherischen Ölen gebräuchlichen aggressiven Reagenzien auch hier zu verwenden, verliefen positiv. Weder Antimon-(III)- und Antimon-(V)-chlorid[5], noch Anisaldehyd–Schwefelsäurereagens führten zu einer Beschädigung des Papieres. Die dabei nötigen Temperaturen bis 90° wurden von dem Papier vertragen. Wegen seiner Empfindlichkeit besonders hervorgehoben sei die Anfärbung ungesättigter Bestandteile mit Osmiumtetroxid[10].

Gegenüber den Dünnschichtplatten bietet das Papier den Vorteil, dass empfindliche Reagenzien, die ein nachträgliches Auswaschen erfordern, Verwendung finden können. So reagierten die geradkettigen Aldehyde wesentlich empfindlicher mit Feulgens Reagens[10] als mit 2,4-Dinitrophenylhydrazin. Auch liess sich die sehr empfindliche alkalische Kaliumpermanganatlösung zur Detektion heranziehen. Bewährt hat sich die aufeinanderfolgende Anwendung von drei Reagenzien. Zuerst werden die Chromatogramme mit Osmiumtetroxiddampf behandelt, wodurch ungesättigte Verbindungen grau bis schwarz gefärbt werden. Dasselbe Chromatogramm wird anschliessend mit einer Antimon-(V)-chloridlösung besprüht, womit sich die Phenole wie Thymol, Carvacrol und andere Stoffe als rotviolette bis braune Flecke anfärben. Durch Aufsprühen einer 2,4-Dinitrophenylhydrazinlösung[12] werden schliesslich Aldehyde und Ketone als gelbe bis rotorange Flecke sichtbar gemacht. Auf diese Weise ist eine schnelle chemische Differenzierung möglich.

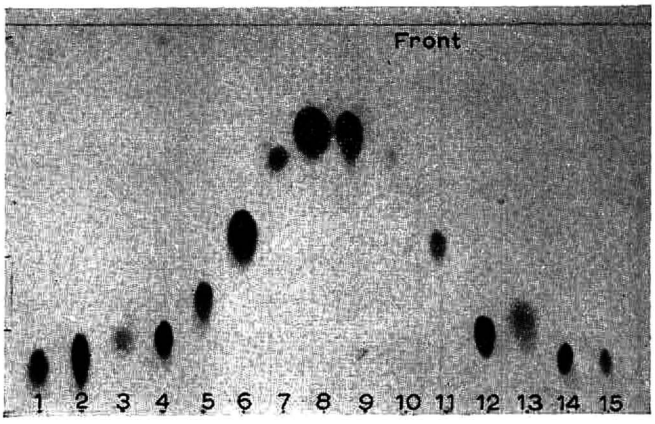


Fig. 3. Aufsteigendes Papierchromatogramm von einzelnen Testsubstanzen. Fliessmittel: 1; Laufzeit: 45 Min.; Anfärbung: Osmiumtetroxid, Antimon(V)-chlorid und 2,4-Dinitrophenylhydrazin nacheinander. 1 = Carvacrol; 2 = Farnesol; 3 = Anisaldehyd; 4 = Caryophyllen + viel Eugenol; 5 = Linalool; 6 = Carvon; 7 = Safrol; 8 = Anethol; 9 = Linalylacetat; 10 = Citronellal; 11 = Citral; 12 = Eugenol; 13 = Zimtaldehyd; 14 = Geraniol; 15 = Thymol.

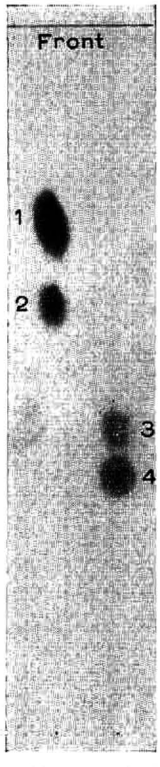


Fig. 4. Absteigend auf mit Paraformaldehyd imprägniertem Papier angefertigtes Chromatogramm zur Trennung von Farnesol(1)–Geraniol(2) und Anisaldehyd(3)–Zimtaldehyd(4). Fliessmittel: 1; Laufzeit: 4 Std. (23°); Anfärbung: Osmiumtetroxid und 2,4-Dinitrophenylhydrazin nacheinander.

Die unterste Nachweisgrenze im sichtbaren Licht liegt im Mittel bei 1 μg. Sie ist z.B. für Thymol und Carvacrol 0.5 μg, für Zimtaldehyd 0.1 μg, für Carvon 0.2 μg und für Linalylacetat 2 μg.

R_F-Werte

Die in Tabelle I angegebenen R_F-Werte stellen Mittelwerte aus je 10 Bestimmungen dar und wurden ohne "Kammerübersättigung" bei 23° ermittelt.

TABELLE I

R_F-WERTE

Verbindung	*Fliessmittel*				
	Aufsteigend				*Absteigend*
	1	*2*	*3*	*4*	*4*
Anethol	0.84	0.85	0.80	0.87	—
Anisaldehyd	0.33	0.30	0.25	0.45	0.43
Carvacrol	0.34	0.30	0.22	0.27	—
Carvon	0.62	0.63	0.55	0.70	—
Citral	0.58	0.56	0.54	0.63	—
Citronellal	0.77	0.75	0.74	0.82	—
Eugenol	0.39	0.38	0.31	0.41	—
Farnesol	0.43	0.53	0.20	0.31	0.73
Geraniol	0.37	0.52	0.24	0.28	0.61
Linalool	0.50	0.50	0.39	0.47	—
Linalylacetat	0.82	0.94	0.78	0.83	—
Safrol	0.79	0.82	0.74	0.78	—
Thymol	0.35	0.33	0.19	0.28	—
Zimtaldehyd	0.37	0.36	0.28	0.49	0.37

EXPERIMENTELLER TEIL

Fliessmittel (Tabelle II)

Als Standardgemisch, geeignet für die meisten ätherischen Öle, kann System 1 bezeichnet werden. Die Systeme 2, 3 und 4 besitzen ebenfalls universelle Anwendbarkeit. Die Gemische 5, 6 und 7 sind für spezielle Trennaufgaben heranzuziehen.

TABELLE II

ENTWICKLUNGSSYSTEME

Nr.	*Lösungsmittel*	*Volumenteile*
1	*n*-Heptan–*n*-Hexan–Eisessig	15:15:2
2	*n*-Heptan–Eisessig	15:1
3	*n*-Heptan–Eisessig	20:0.5
4	Toluol–*n*-Heptan	1:1
5	*n*-Heptan–Methylisopropylketon	20:5
6	Cyclohexan–Äthylacetat	97:3
7	Trichloräthylen	

Methodik

Als Chromatographierkammern für die aufsteigende Chromatographie wurden die von HÖRHAMMER UND WAGNER[9] vorgeschlagenen Rundgläser von 5 l Inhalt ver-

wendet. Zur völligen Sättigung der Atmosphäre werden die Wände jeder Kammer mit einem tropfnass mit dem Fliessmittel getränkten Filtrierpapier (37 × 17 cm) ausgekleidet. Das Fliessmittel — etwa 30 ml — befindet sich in einer Petrischale von 12.5 cm Durchmesser.

Für die absteigende Methode werden Glaskammern von 62 cm Höhe, 52 cm Länge und 21 cm Breite benutzt. Auch hier ist mit getränktem Filtrierpapier für eine völlige Sättigung der Kammeratmosphäre zu sorgen.

Zum Auftragen der Substanzlösungen verwendet man am zweckmässigsten fein ausgezogene Glaskapillaren.

Aufzutragende Mengen

Ätherische Öle werden mit *n*-Heptan, Benzol o.ä. im Volumenverhältnis 1 + 9 verdünnt und von dieser Lösung etwa 0.005–0.01 ml in einem möglichst kleinen Fleck oder Strich mit einem Zwischenraum von je 1.5–2 cm aufgetragen.

Reine Testsubstanzen werden 1 + 29 verdünnt und davon etwa 0.001–0.005 ml aufgebracht. Die Testsubstanzen und die ätherischen Öle müssen wasserfrei sein. Eventuell sind sie nachzutrocknen. Chloroform als Verdünnungsmittel erscheint uns nicht geeignet, da eine rasche Zersetzung zahlreicher Bestandteile in den Verdünnungen zu beobachten ist.

Laufzeiten

Aufsteigende Chromatogramme benötigen mit Fliessmittel 1, bei einer Laufhöhe von 15 cm bei 23°, etwa 45 Min., mit Fliessmittel 5, bei einer für die Trennung von Geraniol, Citronellol und Linalool in Citronell-, Geranium- und Melissenölen ausreichenden Höhe von 20 cm, 90 Min.

Eine für die absteigende Methode genügende Laufstrecke von 37 cm wird mit dem Gemisch 1 in 4 Std. zurückgelegt.

Bei einem Rundfilter von 14.5 cm Durchmesser dauert die Entwicklung des Chromatogramms mit dem System 1 bei Verwendung eines Dochtes mittlerer Saugfähigkeit etwa 20 Min.

Nachweisreagenzien

Osmiumtetroxid. Das Chromatogramm wird nach dem Entwickeln und kurzem Abdunstenlassen des Fliessmittels 30–60 Min. lang in eine dicht schliessende Kammer gebracht und darin dem Osmiumtetroxiddampf ausgesetzt, der aus 0.25 g Osmiumtetroxid erzeugt wird.

Antimon(III)-chlorid. Das Chromatogramm wird auf Vorder- und Rückseite mit einer 10%igen Lösung von Antimon(III)-chlorid in Chloroform besprüht bis es feucht erscheint und anschliessend 15 Min. auf 85° im Trockenschrank erhitzt. Ein nachträgliches kurzes Wässern zum Entfernen der freigewordenen Salzsäure ist für die Haltbarkeit des Chromatogramms über längere Zeiträume vorteilhaft.

Antimon(V)-chlorid. 10%ige Lösung von Antimon(V)-chlorid in reinem Tetrachlorkohlenstoff. Es wird wie bei der $SbCl_3$-Lösung besprüht. Die volle Farbintensität der Flecken wird bei Zimmertemperatur innerhalb von 15 Min. erreicht.

2,4-Dinitrophenylhydrazin. 0.1%ige Lösung von 2,4-Dinitrophenylhydrazin in Äthanol, die 1 Vol. % konz. Salzsäure enthält.

Anisaldehyd–Schwefelsäure-Reagenz. 1.5 Volumenteile Anisaldehyd und 1.5

Volumenteile konz. Schwefelsäure, die in 97 Volumenteilen Methanol gelöst sind. Damit wird das Chromatogramm besprüht und anschliessend bei 50° im Trockenschrank bis zum Erscheinen der Flecken getrocknet.

Kaliumpermanganat. Das Chromatogramm wird in eine Mischung aus gleichen Volumenteilen 5 %iger wässriger Kaliumpermanganat- und 2 % iger Sodalösung getaucht. Anschliessend wird in fliessendem Wasser der Reagenzüberschuss ausgewaschen und das Chromatogramm bei 100° im Trockenschrank getrocknet.

Feulgen-Reagenz. Das Chromatogramm taucht man 15 Min. in eine Fuchsin–schweflige Säurelösung, die 0.25 g Fuchsin/l enthält und durch Zusatz von schwefliger Säure gerade entfärbt ist. Der Überschuss an Fuchsin wird mit einer 0.05 *M* wässrigen SO_2-Lösung dreimal je 15 Min. ausgewaschen.

DISKUSSION

Wenn man von der in den letzten Jahren möglich gewordenen Mikroanalyse ätherischer Öle durch die Gaschromatographie absieht, stellt die vorliegende Methode das erste brauchbare Verfahren dar, um ätherische Öle und deren Bestandteile *direkt* auf Papier aufzutrennen und anschliessend durch Densitometrie quantitativ auszuwerten. Ausser der densitometrischen Bestimmung von 2,4-Dinitrophenylhydrazonen verschiedener Aldehyde und Ketone auf Papier durch SCHULTE UND STORP[11–13], machte HEFENDEHL[14] auf der Grundlage der qualitativen Arbeiten von STAHL[4–8] einen ersten Versuch in dieser Richtung auf Dünnschichtplatten. Er bestimmte dabei den Menthofurangehalt in Pfefferminzölen. Es wurde hierbei jedoch die photographische Reproduktion der Chromatogramme herangezogen. Wie schon KAUFMANN UND HENNIG[15] fanden jedoch auch wir, dass dieses auch von MIRAM UND PFEIFER[16] für die Bestimmung von Alkaloiden benutzte Verfahren wegen der Empfindlichkeitsschwankungen des Photomaterials nicht die gewünschte Reproduzierbarkeit aufweist.

In einer kürzlich erschienenen Veröffentlichung von YORK[17] wird wohl die Möglichkeit einer densitometrischen Auswertung von Harzen und Balsamen auf Dünnschichtplatten mittels eines Reflexionsdensitometers berichtet, doch werden keine verfahrenstechnischen Einzelheiten genannt. Gegenüber der Densitometrie auf Papier dürften die Fehlergrenzen wohl höher liegen.

Mit dem von uns entwickelten Papier haben wir dagegen die Möglichkeit, eine densitometrische Bestimmung mit dem bereits in jeder grösseren Klinik bei der Serum-Eiweiss-Analyse bewährten, von GRASSMANN UND HANNIG[18] entwickelten Durchstrahl-Densitometer* auszuführen. Der Chromatographie braucht dabei nicht erst die zeitraubende Herstellung des Trägermaterials und seine Aktivierung voranzugehen. Weitere Vorteile des Papieres gegenüber der Dünnschichtplatte sind die einfachere Dokumentation, die reproduzierbaren R_F-Werte, die einfache Rundfilterchromatographie und die besseren Ergebnisse bei der Trennung der oben genannten Terpenalkohole, sowie die Möglichkeit der absteigenden Chromatographie. Mit der Dünnschichtplatte hat unser Papier die kurze Laufzeit und die gute Trennfähigkeit gemeinsam.

* Das Gerät wird unter dem Namen Elphor-Integraph von der Fa. Dr. Bender & Dr. Hobein, München, Deutschland, vertrieben.

DANK

Wir danken Herrn Prof. Dr. L. HÖRHAMMER, Direktor des Instituts für pharmazeutische Arzneimittellehre der Universität München, für gewährte Unterstützung dieser Arbeit. Unser besonderer Dank gilt den Firmen Dragoco und Haarmann und Reimer in Holzminden, der Fa. Frey und Lau in Hamburg-Bahrenfeld, die uns Proben ätherischer Öle und Testsubstanzen zur Verfügung stellten. Ausserdem bedanken wir uns bei der Fa. J. C. Binzer, Hatzfeld/Eder für die freundliche Überlassung von Papiermustern.

ZUSAMMENFASSUNG

Durch Imprägnierung mit Paraformaldehyd wurde ein stark adsorbierendes Chromatographierpapier erhalten, mit dem es möglich ist, in kürzester Zeit ätherische Öle mit üblichen Fliessmitteln zu trennen. Dabei sind die auf-, absteigende und erstmals mit nicht vorbehandelten Ölen auch die zirkuläre Methode in gleicher Weise einfach durchzuführen. Es ergeben sich reproduzierbare R_F-Werte. Die Trennfähigkeit und die Einheitlichkeit des Papieres erlauben, es für direkte densitometrische Bestimmungen zu verwenden.

SUMMARY

Chromatography paper with strong adsorbing properties was obtained by impregnation with paraformaldehyde. With this paper essential oils can be separated very rapidly using the common solvents. Ascending or descending development is possible, as well as the circular method, this being the first instance of application of the latter method to untreated oils. The R_F values obtained are reproducible. The separating ability and the homogeneity of the paper make it possible to carry out densitometric determinations directly.

LITERATUR

[1] L. HÖRHAMMER, G. RICHTER UND H. WAGNER, *J. Chromatog.*, 10 (1963) 108.
[2] L. HÖRHAMMER, H. WAGNER UND G. RICHTER, *Biochem. Z.*, 331 (1959) 155.
[3] L. HÖRHAMMER UND G. RICHTER, *Biochem. Z.*, 332 (1959) 186.
[4] E. STAHL, *Pharmazie*, 11 (1956) 633.
[5] E. STAHL, *Chemiker Ztg.*, 82 (1958) 323.
[6] E. STAHL, *Parfuem. Kosmetik*, 39 (1958) 564.
[7] E. STAHL, *Pharm. Rundschau*, 1 (1959) 1.
[8] E. STAHL, *Angew. Chem.*, 19 (1691) 646.
[9] L. HÖRHAMMER UND H. WAGNER, *Deut. Apotheker-Ztg.*, 101 (1961) 779.
[10] M. H. HACK, *Biochem. J.*, 54 (1953) 602.
[11] K. E. SCHULTE UND C. B. STORP, *Fette, Seifen, Anstrichmittel*, 63 (1961) 908.
[12] K. E. SCHULTE UND C. B. STORP, *Fette, Seifen, Anstrichmittel*, 57 (1955) 600.
[13] K. E. SCHULTE UND C. B. STORP, *Fette, Seifen, Anstrichmittel*, 58 (1956) 35.
[14] F. W. HEFENDEHL, *Planta Med.*, 8 (1960) 65.
[15] H. P. KAUFMANN UND H. J. HENNIG, *Fette, Seifen, Anstrichmittel*, 63 (1961) 908.
[16] R. MIRAM UND S. PFEIFER, *Pharm. Zentralhalle*, 96 (1957) 457.
[17] H. YORK, *Deut. Apotheker-Ztg.*, 102 (1962) 1163.
[18] W. GRASSMANN UND K. HANNIG, *Z. Physiol. Chem.*, 290 (1952) 1.

ION-EXCHANGE RESIN PAPER FOR SEPARATION OF THE ACIDIC URINARY METABOLITES OF NOREPINEPHRINE-2-^{14}C IN HUMAN SUBJECTS*

GEORGE F. BAJOR AND WILLIAM GILBERT CLARK

Psychopharmacology Research Laboratories, Veterans Administration Hospital, Sepulveda, Calif., and Department of Biological Chemistry, Medical Center, University of California, Los Angeles, Calif. (U.S.A.)

(Received September 30th, 1963)

In the course of studies on the metabolism of labeled norepinephrine in essential hypertensive patients, a method has been developed for the separation of the acidic metabolites using ion-exchange paper, which was thought worthwhile to report here, in addition to preliminary results obtained in normotensive and hypertensive individuals.

GOODALL *et al.*[1], KIRSHNER *et al*[2,3] and MASUOKA *et al.*[4] have described methods for separating and determining the metabolites of norepinephrine-^{14}C by column chromatography, using CG-50 and Dowex-1 ion-exchange resins and alumina columns.

The method reported here is essentially an adaption from KIRSHNER *et al.* and GOODALL *et al.*[1-3] but substituting Dowex-1 ion-exchange resin-impregnated paper for Dowex-1 columns. Since ion-exchange resin-loaded papers have become available, several reports have appeared in the literature, showing successful application of these papers in the separation of a wide variety of organic and inorganic compounds (see Reeve-Angel brochures for literature, 9 Bridewell Place, Clifton, N.J.).

METHOD AND RESULTS

DL-Norepinephrine-2-^{14}C-acetate (purchased from the Commissariat à L'Énergie Atomique, Gif-sur-Yvette, Seine-et-Oise, France, who synthesized it by a method developed in this laboratory by HOWTON, MEAD AND CLARK[5]) 20 mC/mmol, 46 μC (370 μg, total) was sterilized by the millipore membrane filter technique and infused intravenously in 500 ml 5% dextrose over a 30 min period (equivalent to 0.09 μg/kg/min L-norepinephrine), into 6 normotensives and 6 frank essential hypertensives without abnormal renal function. The urines were collected in containers with acetic acid at hourly intervals from the start of infusion for 6 h, then every 6 h for 24 h. After removing an aliquot of the urine specimen for determining total radioactivity relative to that infused, a 5 ml aliquot was adjusted to pH 6.1 with NH_4OH and passed through a 3 × 1 cm column of Amberlite CG-50 resin, Type 2, 200–400 mesh (Rohm and Haas, Co., Philadelphia), in the NH_4^+ form at pH 6.1 at the rate of 4–5 drops/min.

* Supported in part by U.S. Public Health Service (MY-3663:H-5329), Los Angeles County Heart Association (225), American Heart Association (60-G-27), and National Association Mental Health.

The resin was previously cycled and converted to the NH_4^+ form by the method of HIRS, MOORE AND STEIN[6], *cf.* KIRSHNER AND GOODALL[3]. The column was washed with 15 ml H_2O and after removing an aliquot from the combined effluent and wash for estimating the radioactivity of the unretained neutral and acidic metabolites, the effluent plus wash was lyophilized nearly to dryness. The column, retaining norepinephrine and normetanephrine, was eluted with 20 ml 0.5 *M* acetic acid, made to 25 ml, and 0.1 ml aliquots counted in a Packard Tri-Carb liquid scintillation spectrometer in 10 ml of phosphor consisting of 30% absolute ethanol and 70% toluene containing 0.4% of 2,5-diphenyloxazole (PPO) and 0.01% (1,4-bis-2-[5-phenyloxazolyl]-benzene) (POPOP).

The results were expressed in disintegrations per minute by use of an internal standard. The lyophilized CG-50 effluent plus wash, containing the acidic metabolites, was made to 0.5 ml volume with H_2O and a 10–50 μl aliquot spotted in six steps, drying with nitrogen at each step, along with 3-methoxy-4-hydroxymandelic (MOMA) and 3,4-dihydroxymandelic acid (DOMA) standards, on 18 × 22 in. sheets of Reeve-Angel Type SB-2 (Dowex-1 or IRA-400) ion-exchange resin paper (Reeve-Angel & & Co., Inc., Clifton, N. J.) in the acetate form. To avoid streaking it is necessary to convert it to this form from the chloride form in which it is supplied. This was done by allowing 4 *M* ammonium acetate–acetic acid, pH 6.5, to wash through the paper by descending development in a Chromatocab for several days or until the buffer dripping off the bottom of the paper showed only slight cloudiness with $AgNO_3$. The wet paper then was placed in a large enameled pan, washed twice with distilled H_2O and dried. The acid metabolites and standards, as many as 12 per sheet, were developed with 10.0 *M* ammonium acetate, pH 5.0 by the descending technique in a Chromatocab at constant temperature (25°) for approximately 26 h, or until the solvent front was 39–40 cm from the origin.

It was found that development with 10 *M* gave the best separation of standards and least streaking, with the R_F values of DOMA and MOMA being 0.35 and 0.53 respectively; in 4 *M* they were 0.13 and 0.29; in 1 *M*, 0.04 and 0.07 respectively. The paper was dried overnight in air, and the spots, located readily under ultraviolet light, marked and a tracing of the chromatogram made. The paper then was cut into 2 cm wide strips which were taped end-to-end and scanned in a Vanguard strip-scanner (Vanguard Instrument Co., La Grange, Ill.) to locate the radioactive peaks. For quantitative estimation of activity, the 2 cm longitudinal strips were cut into 1 cm wide segments and counted directly in the liquid scintillation spectrometer in the toluene phosphor without ethanol. No radioactivity eluted off, nor were there problems of orientation of the paper toward the phototube, as reported by WANG AND JONES[7] and LOFTFIELD AND EIGNER[8], because the papers are small enough to fit into the counting vials without folding, and the entire paper size showed no relationship to counting rate, which confirms the findings of BOUSQUET AND CHRISTIAN[9]. Approximately 40% counting efficiency was obtained, using an internal standard.

The results obtained by use of the resin paper compare favorably with the results from a Dowex-1 × 2 resin column, as shown in Figs. 1 and 2. These results were obtained from a urine specimen obtained 1 h after the infusion of norepinephrine-2-^{14}C, as described above, into a normotensive individual. Note that the solvent front is equivalent to the initial fractions from the column. The peaks from the column have been partially identified by GOODALL *et al.*[1]; *cf.* KIRSHNER *et al.*[2], the first as nor-

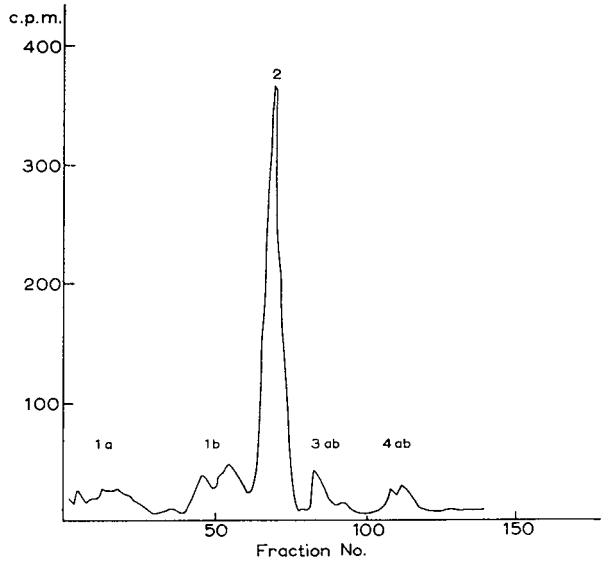


Fig. 1. Separation of metabolites of norepinephrine-2-^{14}C on an ion-exchange column. 1a = normetanephrine conjugate; 1b = unknown; 2 = MOMA; 3a, 3b = DOMA; 4a, 4b = unidentified.

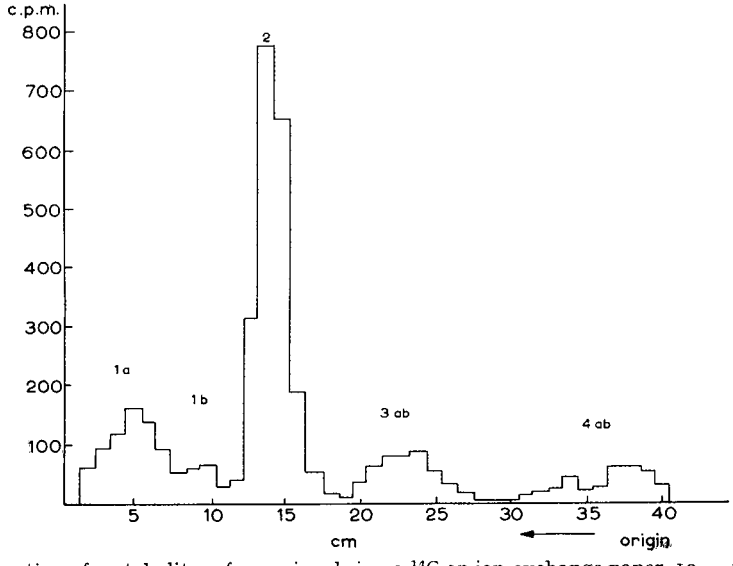


Fig. 2. Separation of metabolites of norepinephrine-2-^{14}C on ion-exchange paper. 1a = normetanephrine conjugate; 1b = unknown; 2 = MOMA; 3a, 3b = DOMA; 4a, 4b = unidentified.

metanephrine conjugate, followed by a small unknown peak (1b), the largest peak (2) as MOMA, followed by DOMA (3a + 3b) and several small unidentified peaks (4a + 4b). The percentage of radioactivity under each peak from the column and the ion-exchange paper are compared in Table I.

TABLE I

PERCENTAGE RADIOACTIVITY UNDER EACH PEAK

Peak No.	*1a and 1b*	*2*	*3a and 3b*	*4a and 4b*	*Total (%)*
Column	27.2	54	7	6.7	94.9
Paper	21.5	55	11	7	94.5

The advantages of the ion-exchange paper over the column method are: shorter time, elimination of fraction collection, and possibility of running up to 12 samples simultaneously.

There was no significant difference between the hypertensives and normotensives in the labeled peaks found, whether compared on the basis of urinary creatinine, for each time of collection, or totalled. This is in agreement with the reports of SJOERDSMA[10], SATO *et al.*[11], GITLOW, *et al.*[12] and others, that apparently there is no defect in the catabolism of *exogenously* administered norepinephrine in hypertension. However, before it can be concluded unequivocally that a defect in the catabolism of endogenous catecholamine may be causally related to the etiology of essential metabolism, a careful study must be made of all endogenous metabolites present. This has as yet not been accomplished, although the elegant studies of LABROSSE *et al.*[13] have accounted for most of the major ones from exogenously administered ^{3}H-labeled material.

SUMMARY

The acidic urinary metabolites of norepinephrine-2-^{14}C were rapidly and conveniently separated and quantitatively determined by first removing the amines on a CG-50 column, which can be eluted and measured. The unretained normetanephrine conjugate, 3-methoxy-4-hydroxymandelic acid, 3,4-dihydroxymandelic acid and several unknowns are separated on Dowex-1 ion-exchange resin-impregnated paper by developing in the descending direction with 10 *M* ammonium acetate. After scanning to locate the areas, the radioactive areas are cut out and counted directly in the liquid scintillation counter.

REFERENCES

1 McC. GOODALL, N. KIRSHNER AND L. ROSEN, *J. Clin. Invest.*, 38 (1959) 707.
2 N. KIRSHNER, L. TERRY AND D. D. POLLARD, *Arch. Intern. Pharmacodyn.*, 131 (1961) 421.
3 N. KIRSHNER AND McC. GOODALL, *J. Biol. Chem.*, 226 (1957) 207.
4 D. T. MASUOKA, W. DRELL, H. F. SCHOTT, A. F. ALCARAZ AND E. C. JAMES, *Anal. Biochem.*, 5 (1963) 426.
5 D. R. HOWTON, J. F. MEAD AND W. G. CLARK, *J. Am. Chem. Soc.*, 77 (1955) 2896.
6 C. H. W. HIRS, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 200 (1953) 493.
7 C. H. WANG AND D. E. JONES, *Biochem. Biophys. Res. Commun.*, 1 (1959) 203.
8 R. B. LOFTFIELD AND F. EIGNER, *Biochem. Biophys. Res. Commun.*, 2 (1960) 72.
9 W. F. BOUSQUET AND J. E. CHRISTIAN, *Anal. Chem.*, 32 (1960) 722.
10 A. SJOERDSMA, *Circulation Res.*, 9 (1961) 743.
11 T. SATO, K. YOSHINAGA, Y. WADA, N. ISHIDA AND C. ITOH, *Tohoku J. Exptl. Med.*, 75 (1961) 151.
12 S. E. GITLOW, M. MENDLOWITZ, S. KHASSIS, F. COHEN AND J. SHA, *J. Clin. Invest.*, 39 (1960) 221.
13 E. H. LA BROSSE, J. AXELROD, I. J. KOPIN AND S. S. KETY, *J. Clin. Invest.*, 40 (1961) 253.

PAPER PARTITION CHROMATOGRAPHY OF RIBOFLAVIN DECOMPOSITION PRODUCTS. THE ACTION OF SOME REDUCING AND OXIDIZING AGENTS ON RIBOFLAVIN SOLUTIONS*

S. SVOBODOVÁ-LEBLOVÁ, J. V. KOŠTÍŘ AND I. M. HAIS

Department of Biochemistry, Charles University of Prague and Faculty of Medicine, University of Hradec Králové (Czechoslovakia)

(Received September 30th, 1963)

Riboflavin is generally reported to be stable in the presence of oxidizing agents[5]. It does not undergo any changes even under the influence of such strong oxidants as nitrous acid, hydrogen peroxide or bromine, but it is attacked by chromic acid[6]. Treatment with potassium permanganate in an acid medium is very commonly used for specific removal of fluorescent contaminants in many analytical procedures for the fluorimetric estimation of riboflavin[7]. It would thus be of considerable practical interest to show whether riboflavin itself is resistant to this treatment.

Various reducing agents such as hydrosulfite or molecular hydrogen cause the formation of the reversible dihydro-form (leuco-form[8]). Reversible oxidation and reduction form the basis of the biological activity of riboflavin-containing nucleotides. By a more drastic reduction KARRER AND OSTWALD[9] obtained octahydroflavin, which was converted to hexahydroflavin on exposure to air.

Our work[4] was mainly concerned with the paper chromatographic pattern of the photolytic products of riboflavin. For comparative purposes the action of reductants and oxidants was also studied. Some of the spots which appear under the influence of hydrogen peroxide have already been mentioned in a preliminary communication by HAIS AND PECÁKOVÁ[1]. Although our study was mainly descriptive in the present stage, it may perhaps yield some points of departure for further study which would explain our observations.

The system of nomenclature, materials and methods used in the present paper has already been described[4]. The most commonly used solvent system was the butanol–acetic acid–water mixture (4:1:5)[1,4]; the mean R_F values found in this solvent, multiplied by 100, together with the color of the fluorescence of the corresponding spot, form the basis of the nomenclature of the spots[4].

EXPERIMENTAL

In order to investigate photolysis in an alkaline medium, riboflavin was dissolved in 0.1 *N* and 0.2 *N* alkali hydroxide. The study of photolysis in neutral and acid media was carried out by mixing the alkaline solution of riboflavin with an amount of acid necessary to obtain the desired pH. For pH values less than 3, hydrochloric acid was used, for pH 4.7, acetic acid.

* For previous communications in this series, see Refs. 1, 2, 3 and 4.

1. *Treatment with thiosulfate and sulfite*

(*a*) *Without light*

On addition of a solution of sodium sulfite or sodium thiosulfate, alkaline, neutral and acid solutions of riboflavin exhibit only a riboflavin spot on paper chromatograms, even if kept in the dark for five days.

(*b*) *With light*

Samples containing both sulfite or thiosulfate, in either alkaline or acid medium, were exposed to daylight for 24 h; riboflavin was then accompanied by very faint 12 CX and 16 CX spots. In an alkaline medium, lumichrome was also detectable after five days. In a neutral medium, the simultaneous action of sodium thiosulfate and light led to the disappearance of the riboflavin spot, and no other spot, detectable in either visible or ultraviolet light, appeared.

2. *Treatment with potassium permanganate*

The influence of potassium permanganate on an alkaline, neutral and acid solution of riboflavin was tested, both in presence and absence of visible light.

(*a*) *Without light*

When potassium permanganate (0.05 *M*) is mixed with a 1 % riboflavin solution (1:1), all fluorescent spots, including riboflavin and some spots with a bluish fluorescence disappear even in the dark. This also occurs in alkaline, neutral and acid solutions of riboflavin.

When a 0.005 *M* solution of potassium permanganate is used, the changes which take place in the dark during the first day of treatment resemble the course of photolysis: acid samples show a weak 27 CX spot[4] and a very weak spot at the position usually occupied by lumichrome (R_F 0.69); in neutral solutions, lumiflavin (50 CX) and lumichrome (69 K) spots are accompanied by a weak 27 CX spot; alkaline samples have a very intense 27 CX spot together with weak lumiflavin (50 CX) and lumichrome (69 K) spots (Fig. 1).

Summarizing, 0.005 *M* $KMnO_4$ without light causes the appearance of a 27 CX spot (most intense in an alkaline medium), a lumiflavin spot (only in alkaline and neutral media) and a lumichrome spot (in all three cases).

No decomposition of riboflavin can be detected with 0.001 *M* permanganate in the dark, irrespective of pH.

(*b*) *With light*

When alkaline, neutral or acid solutions of riboflavin with 0.05 *M* potassium permanganate are exposed to light under the conditions described previously[4], the riboflavin spot disappears during the first four hours. The only fluorescent spots are 39 KI (a bluish purple spot, which is also formed by the action of this concentration of $KMnO_4$ on lumichrome[2]) and small, very weak yellowish spots in a position with an R_F value less than that of riboflavin.

0.005 *M* and 0.05 *M* concentrations of potassium permanganate do not modify appreciably the course of photolysis of riboflavin in neutral and acid samples during the first hours. In an alkaline medium considerably more 27 CX and less lumiflavin (50 CX) are formed than without the acid ion of permanganate.

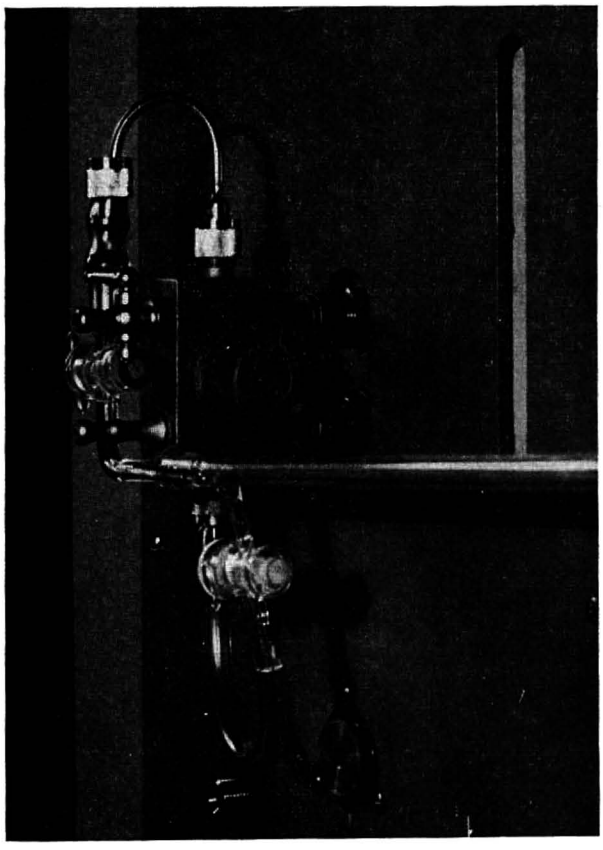


Fig. 4. The manifold for the introduction of samples.

since the stream of helium which is to carry CS_2 into the column must be connected while the cell is still very cold, to avoid evaporation of any water condensed in the cell. Introduction of water vapour into the column would give rise to a peak close to that of the CS_2, so that the latter could not be perfectly zeroed.

If, on the other hand, the temperature of the cell remains too low, the CS_2 may not be completely vaporised. It is therefore advisable to allow the cell to warm up for 2 min after its removal from the Dewar flask before the carrier gas is passed in. About 1 min after connecting the carrier gas, the CS_2 cell is again cut off from the analytical system, to avoid contamination of the column with evaporating water.

Disregarding the limits of sensitivity of the apparatus and of the planimetric measurements, it can be stated with considerable confidence that the method does not give appreciable errors.

In addition to the work with pure water-soluble sodium dithiocarbamate, the method was also tried out on the corresponding zinc, manganese, and iron salts. Since these are insoluble in water, very dilute aqueous suspensions of these compounds were prepared, adding a few drops of a wetting agent (polyglycol esters) to facilitate the dispersion process.

accompanied by the 54 KI spot. The decomposition of alkaline solutions, under the influence of both hydrogen peroxide and light, yields a rich display of 15–25, 54 KI and lumichrome (69 K) spots (Fig. 2).

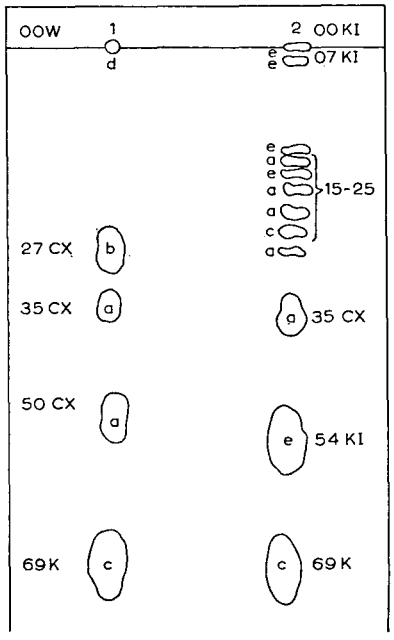


Fig. 2. The influence of hydrogen peroxide on an alkaline solution of riboflavin in presence of visible light: 1. an alkaline solution of riboflavin; 2. an alkaline solution of riboflavin with hydrogen peroxide. The spot fluorescence: a yellow, b weak yellow, c weak blue, d white, e violet-blue.

Lumiflavin (50 CX) is thus mostly missing in the products of alkaline photolysis with hydrogen peroxide, whereas it is present when photolysis takes place without hydrogen peroxide. When 30% hydrogen peroxide was added in a ratio of 1:1 to already photolyzed alkaline samples, the complex 15–25 spots appeared after two days of exposure to daylight, but the intensity of the lumiflavin spot did not diminish appreciably. It follows that lumiflavin (50 CX) is not formed in an alkaline medium through photolysis in the presence of hydrogen peroxide, but that, if already formed, it is not decomposed by hydrogen peroxide.

DISCUSSION

Our results show, in accordance with previous authors, that riboflavin is protected against photolysis by reducing agents. If the sample is applied to the paper in the presence of air, the leuco-form, which is believed to be resistant to photolysis, is oxidized during the handling of the sample for chromatography and unchanged riboflavin is then found on the chromatogram.

The disappearance of any fluorescent spot by the action of sodium thiosulfate and light in a neutral medium is very surprising and merits further investigation.

We could not substantiate the common opinion that riboflavin is not attacked

by oxidants; potassium permanganate causes very drastic decomposition, even in darkness. There can, nevertheless, be no objection to treating analytical samples with permanganate in an acid medium in the light of our results, as we have found that the concentrations used in common fluorimetric analytical procedures do not cause significant losses of riboflavin in acid solution.

It is interesting to note that permanganate itself causes similar qualitative changes to those encountered after irradiation.

The appearance of the intermediate 27 CX seems to be favored by oxidation, as it is encountered even in an acid medium, and its spot is stronger in alkaline medium than without permanganate. Whitby's flavin, whose chromatographic behavior resembles that of 27 CX, is also formed under conditions which suggest oxidation.

Hydrogen peroxide produces some very complicated pictures. Various yellow and bluish purple spots appear, with very irregular R_F values.

54 KI is regularly found in alkaline solutions. After irradiation in an alkaline solution it is interesting to note the absence of lumiflavin (50 CX) and of 27 CX, which is a common precursor of both lumiflavin and lumichrome[4]. Both these substances, nevertheless, are resistant to the action of hydrogen peroxide[3,4], and would not be decomposed if they had been formed.

SUMMARY

The influence of some reducing and oxidizing agents on riboflavin solutions was studied, both in the dark and after irradiation. Sodium sulfite and sodium thiosulfate (except sodium thiosulfate in a neutral medium) seem to protect riboflavin against photolytic decomposition. Riboflavin is attacked by permanganate. The products formed resembled those formed on photolysis. The formation of 27 CX seems to be favored. With hydrogen peroxide, a 54 KI spot and a series of 15–25 spots, with a yellow or bluish-purple fluorescence, are formed. The formation of 27 CX and lumiflavin by alkaline photolysis is inhibited by the presence of hydrogen peroxide.

REFERENCES

1 I. HAIS AND L. PECÁKOVÁ, *Nature*, 163 (1949) 768.
2 I. HAIS AND S. SVOBODOVÁ, *Chem. Listy*, 45 (1951) 25.
3 S. SVOBODOVÁ, *Chem. Listy*, 45 (1951) 225.
4 S. SVOBODOVÁ, I. HAIS AND J. KOŠTÍŘ, *Chem. Listy*, 47 (1953) 205.
5 R. KUHN, H. RUDY AND TH. WAGNER-JAUREGG, *Ber.*, 66 (1933) 1950.
6 U. BĚRĚZOVSKIJ, *Usp. Khim.*, 18 (1949) 737.
7 E. KODICEK, *Analyst*, 72 (1947) 385.
8 H. RUDY, *Fortschritte der Chemie organischer Naturstoffe*, 2 (1939) 60.
9 P. KARRER, *Ueber die Chemie der Flavine, Ergebnisse der Vitamin und Hormonforschung*, Thieme, Leipzig, 1934.

COUNTERCURRENT ELECTROPHORESIS ON PAPER

VIII. THE NATURE OF THE SO-CALLED "DECREASE IN RELATIVE THERMODYNAMIC ACTIVITY" IN PAPER ELECTROPHORESIS*

JIŘÍ VACÍK AND MARIE JAKOUBKOVÁ

Department of Physical Chemistry, Charles University, Prague (Czechoslovakia)

(First received February 1st, 1963)
(Modified September 1st, 1963)

INTRODUCTION

In addition to the concept whereby the influence of the paper on the electrophoretic mobilities of substances is characterised by a tortuosity factor and a separation function[1-5], there is also a concept of "barrier effect" introduced by McDONALD[6]. According to this theory, cellulose fibers act as a barrier to migrating particles. The slowing down of the particles is proportional to the number of barriers, characterised by the value "concentration of paper in the solution", P. This term is determined by the ratio of the weight of paper to the weight of solution. For quantitative expression of this idea, a quantity "relative thermodynamic activity" a_{ct} was introduced, and has unit value for solutions containing no paper ($P = 0$). The presence of paper in the solution ($P > 0$) produces a decrease in the quantity a_{ct}. The values of the quantity a_{ct} of the ions are given by the ratio of the conductivity K of the system with paper ($P > 0$) to the conductivity K_0 of the same system without paper ($P = 0$)[6]. In addition according to ref. 6,

$$U_0 = U_{cor}/a_{ct} \qquad (1)$$

where U_0 is the electrophoretic mobility in the free solution and U_{cor} the macroscopic electrophoretic mobility on paper corrected for electro-osmosis.

MARBACH[6] estimated the "relative thermodynamic activity" from measurements of conductivity. He measured the conductivity of the solution in a vessel which was gradually filled with chromatographic paper. The measured conductivity values were multiplied by the ratio ϑ of the volume of the vessel to the volume of solution actually present in the vessel. From this were obtained results corresponding to the same amount of solution. He calculated further the quantity a_{ct} and plotted the values obtained against the ratio P. A falling straight line resulted. Such results were obtained for papers free of conducting impurities and with solutions of specific conductivities $\varkappa > 4.10^{-4}\Omega^{-1}\text{cm}^{-1}$. When $\varkappa < 4.10^{-4}\Omega^{-1}\text{cm}^{-1}$ (*e.g.* for 0.001 N KCl) MARBACH found even for washed papers (ref. 6, *e.g.* Fig. 24) an increase of specific conductivity with the ratio P. In these cases he did not plot the dependence of a_{ct}

* For Part VII, see ref. 5.

on P. The decrease of "relative thermodynamic activity" obviously did not occur in this case. This purely experimental correlation of U_0 and U without physical analysis was criticized by WALDMANN-MEYER AND SCHILLING[7].

In order to determine the physical meaning of the quantity a_{ct}, the conductivity in MARBACH's experimental arrangement was measured. The cross section of the vessel was denoted by A, the cross section and the weight of liquid in the paper pores by a_1 and g_1 respectively and the cross section and the weight of the liquid in the vessel apart from the paper by a_2 and g_2. For the conductivity of a pure liquid, K_0, and for the conductivity when paper is present, K, the following equations may be written:

$$K_0 = \varkappa_0 A/L; \qquad K = \varkappa_0[a_2 + (a_1/f)]/L \tag{2}$$

where L is the length of the vessel, f the tortuosity factor and $\varkappa_0$ the specific conductivity of the given liquid. If it is assumed that the conductivity $\varkappa_0$ is not influenced by the presence of the paper, it follows that:

$$a_{ct} = K\vartheta/K_0 = 1 - [a_1\vartheta\,(1 - 1/f)/A] \tag{3}$$

because $\vartheta = A/(a_1 - a_2)$. In addition, where H represents the weight of the water already filling all the pores of 1 g of the paper calculated on the dry paper and neglecting the swelling water, and since $P = g_p/(g_1 + g_2)$ (g_p is the weight of paper in the vessel) it follows that $a_1\vartheta/A = g_1/(g_1 + g_2) = g_1P/g_p = HP$. For the quantity a_{ct}, therefore,:

$$a_{ct} = 1 - HP + (HP/f) \tag{4a}$$

If the specific conductivity of the solution changes from the value $\varkappa_0$ to the value $\varkappa_b$ when paper is present, then:

$$a_{ct} = \varkappa_b\,[1 - HP + (HP/f)]/\varkappa_0 \tag{4b}$$

The specific conductivity of the solution changes from $\varkappa_0$ to $\varkappa_b$ for different reasons. When this change is due to the washing out of soluble conducting impurities from the paper the total bulk-conductivity $\varkappa_b$ is given by the sum of the original value, $\varkappa_0$, and of the conductivity of the impurities, $\varkappa_i$ ($\varkappa_b = \varkappa_0 + \varkappa_i$). The interaction of a dissolved substance with the paper (*e.g.* the adsorption of a part of the dissolved conducting substance by the paper or an exchange adsorption of ions of different conductivity on the paper) can also change the original value of the conductivity. Furthermore if, in dilute solutions, the surface-conductivity $\varkappa_s$ in the paper pores is taken into consideration, as well as the bulk-conductivity $\varkappa_b$ (ref. 8) then:

$$K = [(\varkappa_b + \varkappa_s)\,a_1 + \varkappa_b a_2 f]/fL$$

and:

$$a_{ct} = \varkappa_b\,[1 - HP + (HP/f)]/\varkappa_0 - \varkappa_s HP/\varkappa_0 f \tag{4c}$$

Equations (4a, 4b, 4c) are valid for $HP \geqslant 1$, *i.e.* the given experimental conditions. Under the normal conditions of electrophoretic experiments HP is nearly equal to unity. Then:

$$a_{ct} = (\varkappa_b + \varkappa_s)/\varkappa_0 f \tag{5}$$

If the concentration of the solution is large ($\varkappa_i, \varkappa_s \ll \varkappa_0$) and if no interaction takes place between the dissolved substance and the paper ($\varkappa_b = \varkappa_0$), the term a_{ct} becomes the reciprocal of the tortuosity factor and in this case $a_{ct} < 1$. If there is an interaction leading to decrease in specific conductivity and if the concentration of the solution is high, then $\varkappa_b < \varkappa_0$ and $a_{ct} < 1$ again. But when the concentration of the solution is small ($\varkappa_0$ does not differ much from $\varkappa_i$ and $\varkappa_s$) and the interaction with the paper leading to decrease in the specific conductivity is not too large ($\varkappa_b$ does not differ too much from $\varkappa_0$ and f varies from 1.1 to 1.4—see Table I) then $a_{ct} > 1$. When the interaction with the paper leads to the increase of specific conductivity ($\varkappa_b > \varkappa_0$) (exchange adsorption) then it is possible that $a_{ct} > 1$ for concentrated as well as dilute solutions.

The physical meaning of the quantity a_{ct} is consequently far from clear. It is furthermore evident that this quantity is not suitable for correlation of the values U_0 and U_{cor} by equation (1). According to this equation a_{ct} should always be smaller than unity ($a_{ct} < 1$), because in fact U_0 is always greater than U_{cor}. This, however, obviously contradicts the conclusions of eqn. (5).

It is, however, evident, that it is possible to use conductivity measurements of this kind for the estimation of the values of the quantities on the right hand side of eqn. (5). When the solution does not contain any substance which could interact with the paper (in the sense of chromatographic distribution), the difference between the values $\varkappa_b$ and $\varkappa_0$ may be ascribed to impurities in the paper and it is possible to calculate their conductivity $\varkappa_i$. (For $HP = 1$, $\varkappa_i$ becomes the specific conductivity of impurities from the given sample of paper dissolved in the amount of water which just fills all its pores.) On the other hand if the paper is free from impurities, but the solution contains substances which can interact with the paper, it is possible to find the corresponding distribution isotherm.

EXPERIMENTAL

Measurements were performed with Whatman No. 1, 2, 3, 4, 31, 52, Schleicher & Schüll, No. 604, 2043A, 2043B and Ederol No. 202 chromatographic papers. Methylene Blue $C_{16}H_{18}N_3Cl$, Neptun Blue $Ca(C_{37}H_{35}N_2O_2S_2)_2$ and freeze-dried albumin (Imuna, Michalany) were studied. All other substances were of analytical reagent purity. The conductivity measurements were performed in the experimental arrangement described by MARBACH[6]. A thermostated conductivity cell of dimensions 57 × 14 × 21 mm was used and resistances were measured with a Tesla RLC bridge.

RESULTS

The dependence of resistance on the amount of paper added was measured. All measurements were carried out using papers with no previous treatment as well as with papers, which had been freed from soluble impurities. The impurities were removed by decanting for two hours and by washing the paper with a solution of a given concentration. The calculated values of the quantity a_{ct} were plotted against the ratio HP (values H for different kinds of chromatographic papers were taken from the previous paper[9]).

The reciprocal values of the quantity a_{ct} for $HP = 1$ from the measurement in

0.1 N KCl solution ($\varkappa_b = \varkappa_0$) corresponding to the tortuosity factor are shown for different chromatographic papers in Table I. The dependence of the quantity a_{ct} on the ratio HP in solutions of different concentrations of potassium chloride for Whatman No. 4 paper are shown in Fig. 1. Similar dependences were obtained also

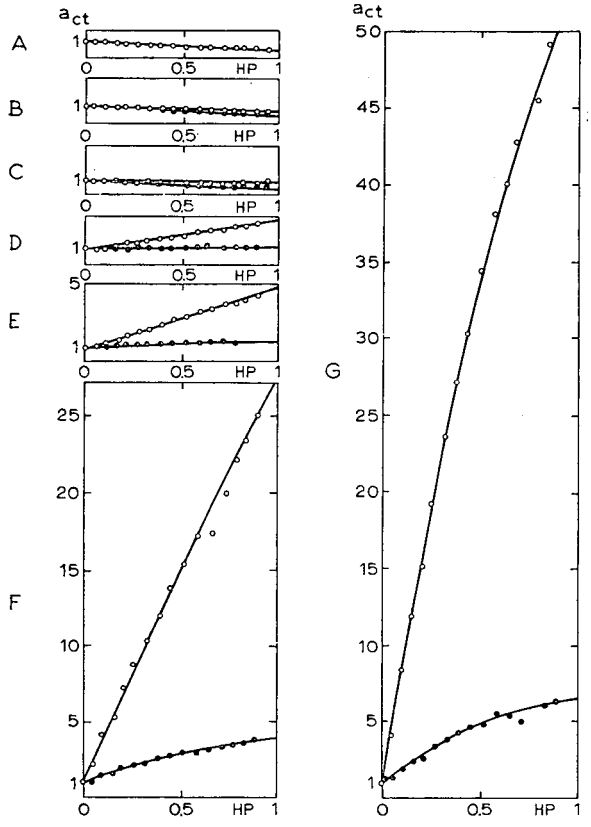


Fig. 1. The dependence of a_{ct} on the quantity HP for different concentrations of potassium chloride solution: A = 0.1, B = 0.01, C = 0.005, D = 0.001, E = 0.0005, F = 0.0001, G = 0.00005 M. ● washed paper, ○ unwashed paper.

for other kinds of chromatographic papers. The surface-conductivity $\varkappa_s$ was calculated from eqn. (5) using the values of a_{ct} which were obtained from the measurements of washed papers ($\varkappa_0 = \varkappa_s$). From the values of a_{ct} obtained from the measurements with papers without previous treatment ($\varkappa_b = \varkappa_i + \varkappa_0$) conductivities of impurities $\varkappa_i$ were calculated and these results are shown in Table I. Whatman No. 4 paper was also measured in different electrolytes; the results of these measurements are shown in Tables I and II.

In order to study the interaction of the substance with paper, solutions were prepared containing different amounts of the interacting substance in the same amount of electrolyte. Solutions obtained in this way had different specific conductivities. Another series of measurements was performed with solutions also containing differ-

TABLE I

TORTUOSITY FACTOR f, SURFACE CONDUCTIVITY $\varkappa_s$ AND CONDUCTIVITY OF IMPURITIES $\varkappa_i$ OF SOME CHROMATOGRAPHIC PAPERS

Paper		Whatman No. 1	2	3	4	31	52	Schleicher & Schüll No. 604	2043A	2043B	Ederol No. 202
f		1.28	1.31	1.31	1.28	1.22	1.25	1.25	1.25	—	1.39
$\varkappa_s \cdot 10^{-5}$ [Ω^{-1}cm^{-1}]		4.64	3.93	3.19	4.80	3.59	2.10	6.76	3.19	3.56	3.05
$\varkappa_i \cdot 10^{-4}$ [Ω^{-1}cm^{-1}]	5.10^{-3} N KCl	2.37	1.37	—	2.56	0.85	—	3.52	2.88	1.95	—
	5.10^{-4} N KCl	2.34	2.25	0.97	2.74	0.93	—	3.61	3.45	2.50	1.96
	5.10^{-5} N KCl	2.17	2.41	1.48	2.90	1.24	3.09	3.81	3.09	2.03	2.65
	Average values*	2.29	2.01	1.22	2.80	1.01	3.09	3.65	3.14	2.16	2.30

* Average values are taken from all measurements performed, not only from those in Table I.

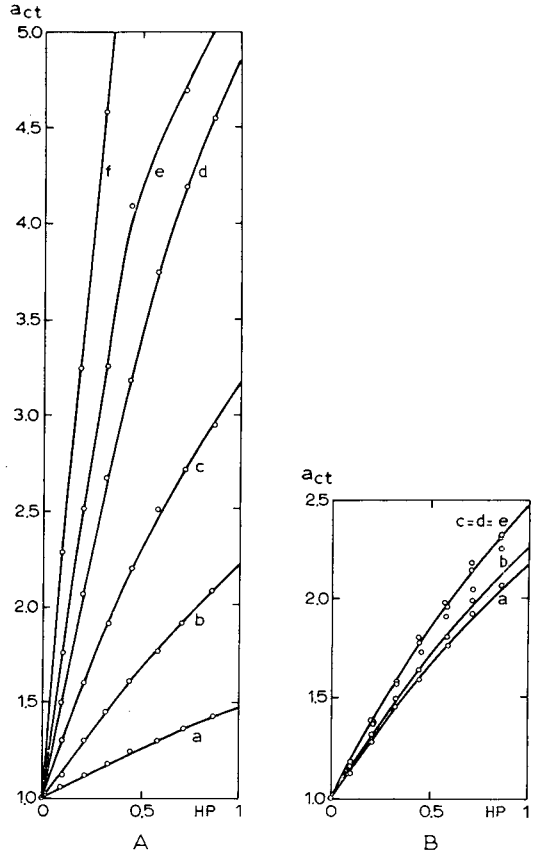


Fig. 2. The dependence of a_{ct} on the quantity HP. (A) Solutions of albumin in standard acetate buffer diluted with water (1:99). Concentration of albumin: a = 1, b = 0.5, c = 0.25, d = 0.1, e = 0.05%, f = buffer without albumin. (B) Analogous solutions with constant conductivity; curve f corresponds to the buffer solution used for dilution.

TABLE II

CONDUCTIVITY OF IMPURITIES $\varkappa_i \cdot 10^{-4}[\Omega^{-1}cm^{-1}]$ FOR WHATMAN NO. 4 PAPER IN DIFFERENT SOLUTIONS

Base solution	*Dilution base solution: water*				*Average values*
	1:1	*1:9*	*1:99*	*1:999*	
0.1 *N* Potassium chloride	—	—	2.84	2.97	2.80
0.1 *N* Acetic acid	2.04	2.95	3.04	2.76	2.70
Veronal buffer[a]	1.57	3.64	2.71	3.19	2.81
Phosphate–borate buffer[b]	—	3.14	2.83	3.59	3.08
Acetic acid–formic acid[c]	—	—	1.04	3.17	2.10

[a] 2.06% veronal-Na, 0.368% barbituric acid.
[b] 0.466% $NaH_2PO_4 \cdot H_2O$, 0.85% $Na_2B_4O_7 \cdot 10H_2O$.
[c] 17.7% acetic acid, 5.1% formic acid.

ent amounts of the interacting substance but adjusted (by changing the concentration of the electrolyte) to the same specific conductivity. The dependence of the quantity a_{ct} on the ratio HP obtained from these measurements is plotted in Figs. 2–4.

DISCUSSION

The advantage of the method described for measuring the tortuosity factor is that it is not obtained from a single measurement, but from a concentration dependence, where random experimental errors can be eliminated graphically. A further advantage is that it is possible also to determine the correcting factor for moistures of paper larger than H.

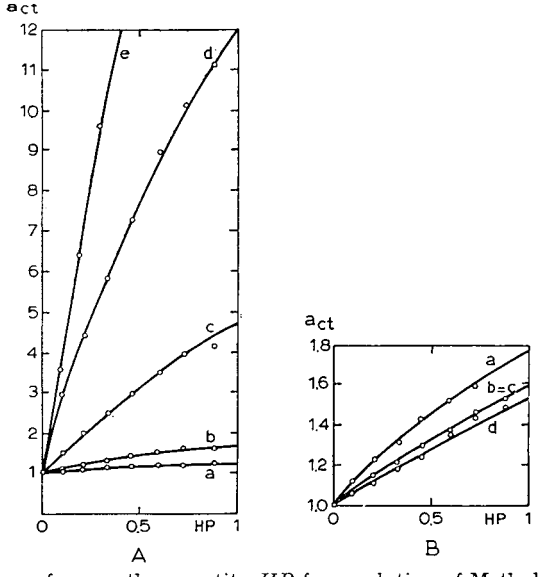


Fig. 3. The dependence of a_{ct} on the quantity HP for a solution of Methylene Blue in 0.0001 *N* acetic acid. (A) Concentration of the dye: a = 0.1, b = 0.05, c = 0.01, d = 0.005%, e = pure acetic acid. (B) Concentration of the dye: a = 0.05, b = 0.025, c = 0.01%, d = pure 0.03 *N* acetic acid. Constant specific conductivity.

It was found that papers contain conducting impurities in such amounts that $\varkappa_i$ for all of them is in the order of $10^{-4}\Omega^{-1}$ cm^{-1}.

According to the literature[8], it was found that surface-conductivity in the pores of the paper is practically independent of the concentration of the electrolyte. Moreover, the surface conductivity of all the papers used in all the electrolytes tested was found to be practically the same, *viz.* of the order $10^{-5}\Omega^{-1}$ cm^{-1}.

The increase in the quantity a_{ct} due to the conductivity of impurities and surface-conductivity is seen in Fig. 1. The influence of impurities and of surface-conductivity is negligible in concentrated potassium chloride, and increases with dilution of the solution. The effect of interaction between the substance and the paper on the value of a_{ct} is clearly seen only under experimental conditions in which the concentration of interacting substance varies, while the specific conductivity of the solution is kept constant (Figs. 2B, 3B, 4B). It is otherwise impossible to determine to what extent the change of the quantity a_{ct} is due to the interaction and to what extent to the increase in bulk-conductivity of the solution. It is, however, shown that a change (increase or decrease) of the quantity a_{ct} due to the interaction is always much smaller than the decrease of a_{ct} exhibited by the increase in the specific conductivity of the solution (Figs. 2A, 3A, 4A).

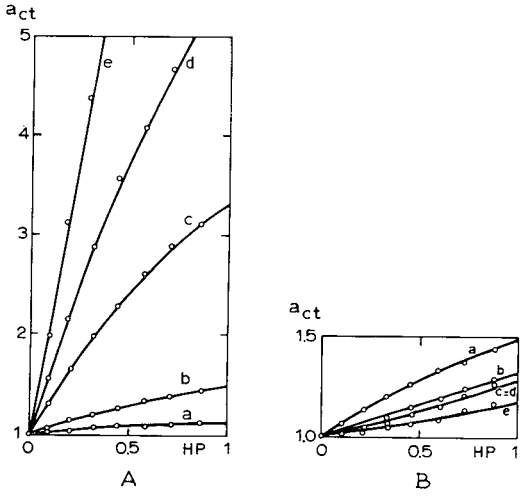


Fig. 4. The dependence of a_{ct} on the quantity HP for a solution of Neptun Blue in 0.0001 N acetic acid. (A) Concentration of the dye: a = 0.1, b = 0.05, c = 0.01, d = 0.005%, e = pure acetic acid. (B) Concentration of the dye: a = 0.05, b = 0.025, c = 0.01, d = 0.005%, e = pure 0.06 N acetic acid. Constant specific conductivity.

It is evident that the interaction of the substance with paper can lead both to a decrease in conductivity of the solution (albumin), and also to its increase (Methylene Blue and Neptun Blue). The main role is played by the substance, which has a direct share in the interaction. In exchange adsorption on paper, where an electric double-layer structure is involved, a cation exchange can be assumed. Methylene Blue therefore exhibits an increase of specific conductivity due to the exchange of the coloured organic cation for the more mobile ion from the outer part of the electric

double-layer. This is also in agreement with the relatively smaller R_F value of Methylene Blue in the given solution (R_F = 0.03–0.3. The R_F values were obtained by frontal chromatography). With Neptun Blue the coloured part is an organic anion which is not adsorbed on the negatively charged cellulose (R_F approx. = 1). In this solution calcium cations take part in the adsorption leading to an increase in $\varkappa_b$. It is seen also that in systems in which the influence of surface conductivity of impurities is eliminated the interaction indicated by conductivity measurements of a given substance need not be the same as that indicated by chromatographic distribution.

SUMMARY

It has been shown that, under special conditions, the so-called "relative thermodynamic activity" can be expressed as the reciprocal value of the tortuosity factor. The value of this "activity" can not only decrease with the "paper concentration", but can also increase to values above unity. It is therefore not possible to consider the term "relative thermodynamic acitivity" as a general characteristic of the system solution–paper. On the other hand changes in electrical conductivity can indicate the interaction of the dissolved substance with paper and/or the presence of impurities. Measurements of this kind lead also to an evaluation of surface conductivity.

REFERENCES

1 H. G. KUNKEL AND A. TISELIUS, *J. Gen. Physiol.*, 35 (1951) 89.
2 R. CRAWFORD AND J. T. EDWARD, *Anal. Chem.*, 29 (1957) 1543.
3 J. T. EDWARD, *J. Chromatog.*, 1 (1958) 521.
4 Z. FIDLER, J. VACÍK, J. DVOŘÁK AND O. GRUBNER, *J. Chromatog.*, 7 (1962) 228.
5 J. SCHRAML, J. VACÍK, O. GRUBNER AND J. DVOŘÁK, *J. Chromatog.*, 9 (1962) 154.
6 H. J. MCDONALD, *Ionography. Electrophoresis in Stabilised Media*, The Year Book Publishers. Inc., Chicago, 1955.
7 H. WALDMANN-MEYER AND K. SCHILLING, *Acta Chem. Scand.*, 13 (1959) 13.
8 I. I. ZHUKOV (ed.), *Elektrokineticheskie svoistva kapillarnykh sistem* (*Elektrokinetic Characteristics of Capillary Systems*), Moscow-Leningrad, 1956.
9 J. VACÍK, O. GRUBNER AND J. DVOŘÁK, *Collection.Czech. Chem. Commun.*, 25 (1960) 625.

COUNTERCURRENT ELECTROPHORESIS ON PAPER

IX. COMPARISON OF ELECTROPHORETIC, CHROMATOGRAPHIC AND STATIC DISTRIBUTION ISOTHERMS*

KAREL PEJŠA, LUDMILA DOBÍHALOVÁ, ZDENĚK FIDLER AND JIRÍ DVOŘÁK

Department of Physical Chemistry, Charles University, Prague (Czechoslovakia)

(First received February 1st, 1963)
(Modified September 1st, 1963)

INTRODUCTION

The preceding parts of this series have shown the agreement of electrophoretic distribution isotherms with isotherms obtained by static measurements[1] and the suitability of the frontal method for the measurement of electrophoretic distribution isotherms[2]. The object of this paper is to show the agreement of all three kinds of distribution isotherms, *i.e.* electrophoretic, chromatographic and static and the influence of the swelling of the stationary phase on the results of the measurement of distribution isotherms. The frontal method[2-4] has been applied to the measurement of chromatographic distribution isotherms.

In order to calculate the values of the distribution isotherm $q\,(c)$ (the amount adsorbed by 1 g of stationary phase) by our method the following quantities are measured: V = volume of the solvent passing through the column during the time t, V_0 = total volume of the column, x_0 = weight of the stationary phase in the column, ρ = density of the stationary phase, c = concentration of the solution. Since the frontal method is involved, the derivation $q'(c) = \mathrm{d}q(c)/\mathrm{d}c = q(c)/c$ and the DE VAULT[5] equation may be written in the form $q(c) = [(\partial t/\partial x)_c w - \alpha]c$. Here α is the pore space. Hence in the case of swelling material, 1 g of which takes up b ml of water we have the expression:

$$q(c) - bc = \left\{\left[\left(\frac{\partial t}{\partial V_0}\right)_c \frac{V}{t} - 1\right] \frac{V_0}{x_0} + \frac{1}{\rho}\right\} c \qquad (1)$$

For static measurement we already used[1] the equation:

$$q(c) - bc = \frac{V^*}{x}(c_0 - c) \qquad (2)$$

where V^* is the volume of the solution, x the weight of the dry adsorbent, c_0 the concentration of the original solution and c the concentration of the equilibrium solution.

Measurement of electrophoretic distribution isotherms for swelling material does not lead to values $q(c)$, but to values $q(c) - bc$. Thus the value of swelling water,

* Part VIII: J. VACÍK AND M. JAKOUBKOVÁ, *J. Chromatog.*, 14 (1963) 456.

b, can not be obtained by any of the methods for measuring the function $q(c)$ nor by their combination. An independent method can be based on the comparison of the volume flow rate (dV/dt, $cm^3 \cdot sec^{-1}$) and the linear flow rate (dL/dt, $cm \cdot sec^{-1}$). The following equation then follows:

$$\frac{dV}{dt} = pS(\alpha - b)\frac{dL}{dt} \tag{3}$$

where S is the width of the paper strip, L the length in the direction of the flow, and p the weight of the paper in $g \cdot cm^{-2}$. The rate dL/dt can be measured by following a boundary on both sides of which the solutions possess the same adsorptive properties. The boundary of sodium phosphate solutions differing only in the radioactivity of phosphorus was used. The measurement mentioned leads to the definition of the value of swelling water as all the water that does not take part in the flow.

EXPERIMENTAL

The temperature was kept at 20° ± 1°, for all measurements. The structural parameters of the chromatographic papers used were taken from the preceding paper[6]. The concentrations are expressed in g/l.

Material

Ground glass (Simax), kieselguhr (Kaznějov), Al_2O_3 (for chromatography, Lachema), polyamide, cellulose (Whatman) and Whatman Nos. 1, 2 and 4 chromatographic papers were used as the stationary phase. Loose materials (except cellulose) were purified by washing and sieve screening and their densities were determined by pycnometry. 0.1 and 1.0 N acetic acid (analytical reagent purity) was used as the mobile phase. The measurement of distribution isotherms was carried out for different dyes and the dye Kashmir Blue T.G. Extra (Bayer, Elberfeld, Germany) was used for reference. The isotope ^{32}P added to non-active Na_3PO_4 was used for measuring the swelling water.

Chromatographic measurements

A burette (10 ml) was filled with the stationary phase. A constant level above the stationary phase was automatically maintained by adding solution from the storage flask. The movement of the front was measured by reading the volume on the burette. The flow of the solvent was determined by measuring the volume of the liquid which passed through the column. The flow rate was kept constant throughout.

Paper chromatography was performed by the descending method in a vapour-saturated space. The movement of the front was read on the scale in units of length. Velocity was then expressed as the volume rate. The flow of solvent was determined by measuring the volume of liquid necessary to keep the level in the upper trough constant.

Static measurements

A paper strip and/or cellulose powder were dried (115°, 4 h). To the weighed amount (x) of paper or cellulose a volume (V^*) of solution was added corresponding to the

ratio V^*/x chosen. The paper was kept with the solution in closed glass vessels for 120 h. The original and final concentrations were determined by spectrophotometry at a suitable wave length.

Measurement of swelling water

This was performed in a wet chamber by means of a Geiger–Müller counting tube GM 30/50B, Tesla. The concentration of Na_3PO_4 was 0.001 M.

RESULTS AND DISCUSSION

An example of the direct results of frontal measurement is given in Fig. 1 and an example of a distribution isotherm for swelling material is given in Fig. 2. The values of $q(c)$ were calculated using $b = 0.66$. In all cases of non-swelling materials, smooth curves were obtained whereas swelling materials resulted in curves with a maximum.

The curves $c_0 - c$ obtained from static measurements deviate from the line $c_0 = c$ under the influence of the transition of the dye into the cellulose phase and under the influence of swelling. In all cases the influence of the transition of the dye dominated and $c < c_0$. An example is given in Fig. 3.

By performing both the measurements described for the dye Kashmir Blue on Whatman No. 4 chromatographic paper these results can be compared with the values obtained electrophoretically in the preceding paper[2] (see Fig. 4). The course of the curves is seen to be identical and the absolute values show a satisfactory agreement.

The swelling water was found to be 0.54, 0.66 and 0.65 in 0.0, 0.1 and 1.0 N acetic acid, respectively (water vapour saturated atmosphere, 20°, Whatman No. 4 paper).

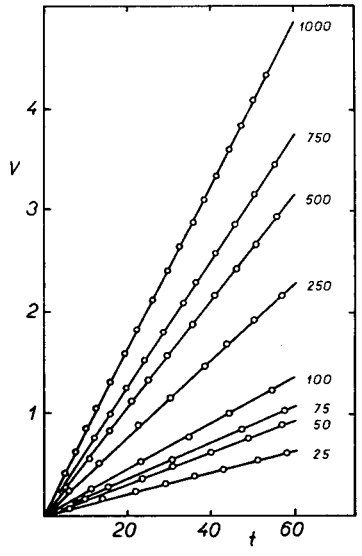


Fig. 1. Movement of the Methylene Blue front (0.1 N acetic acid, Whatman No. 4 paper) at different dye concentrations (indicated on the curves in p.p.m.).

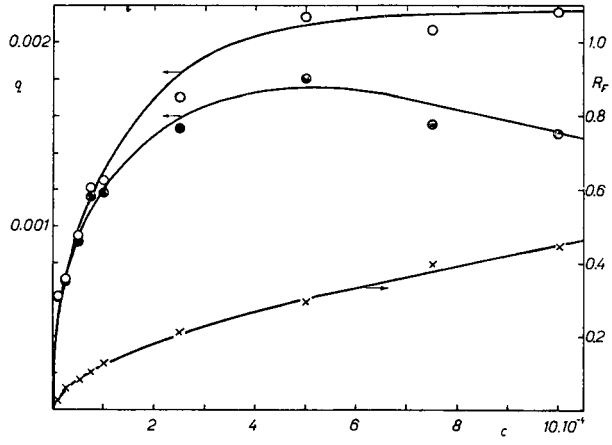


Fig. 2. Chromatographic isotherms $q(c)$ (○), $q(c) - bc$ (●), and the concentration dependence of R_F (×) for Methylene Blue and cellulose powder in 0.1 N acetic acid.

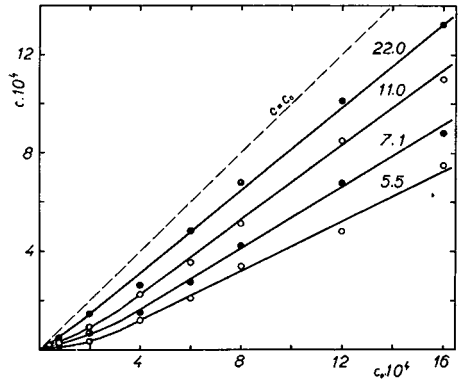


Fig. 3. Static relationship between equilibrium concentration c and original concentration c_0 for Naphthol Black and Whatman No. 4 paper in 0.1 N acetic acid. Ratio V^*/x indicated at the curves.

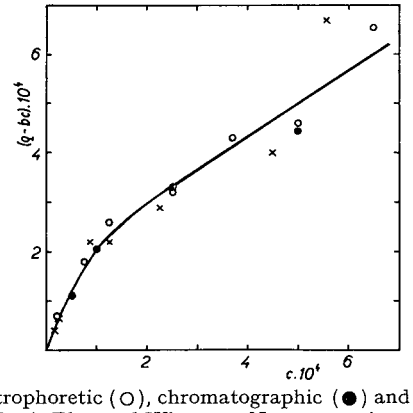


Fig. 4. Comparison of electrophoretic (○), chromatographic (●) and static (×) distribution isotherms for Kashmir Blue and Whatman No. 4 paper in 0.1 N acetic acid.

ACKNOWLEDGEMENT

The authors are indebted to Dr. O. GRUBNER and J. VACÍK for their advice and interest in this work.

SUMMARY

It has been shown that swelling of the stationary phase influences the static and chromatographic measurements of distribution isotherms. If the swelling of paper is disregarded when calculating the adsorbed amount from the results of both static and chromatographic measurements the values obtained are diminished. The distribution isotherm can even show a maximum. The value of swelling water was measured for the conditions used. For measurement of chromatographic distribution isotherms the frontal method was applied. The identity of the electrophoretic, chromatographic and static distribution isotherms was proved using Kashmir Blue in 1.0 *N* acetic acid on Whatman No. 4 chromatographic paper as an example.

REFERENCES

1 Z. FIDLER, J. VACÍK, J. DVOŘÁK AND O. GRUBNER, *J. Chromatog.*, 7 (1962) 228.
2 J. SCHRAML, J. VACÍK, O. GRUBNER AND J. DVOŘÁK, *J. Chromatog.*, 9 (1962) 154.
3 S. CLAESSON, *Arkiv Kemi*, 23 A (1946) 1.
4 F. H. M. NESTLER AND H. G. CASSIDY, *J. Am. Chem. Soc.*, 72 (1950) 680.
5 D. DE VAULT, *J. Am. Chem. Soc.*, 65 (1943) 532.
6 J. VACÍK, O. GRUBNER AND J. DVOŘÁK, *Collection Czech. Chem. Commun.*, 25 (1960) 625.

SEPARATION OF THE RARE EARTH ELEMENTS BY ION-EXCHANGE CHROMATOGRAPHY

I. THE USE OF NH_4^+–H^+ DEVELOPMENT COLUMNS

N. E. TOPP AND D. D. YOUNG*

Department of Scientific and Industrial Research, National Chemical Laboratory, Teddington, Middx. (Great Britain)

(Received September 25th, 1963)

Rare earth cations have similar affinities for ion-exchange materials, and separation methods based on difference in affinity have met with little success[1,2]. Modern methods of separating these elements depend on the use of complexing agents[3]. This method was used for separating and identifying tracer quantities of fission product rare earths[4,5]. The underlying theory of the method was also established. If two rare earth cations, Ln_1^{3+} and Ln_2^{3+} have association constants K_1 and K_2 for a ligand Y^{n-}, then the separation factor between the two elements (K_s) is K_2/K_1[6]. Separation is thus dependent on differences in affinity between the individual rare earth cations and the ligand.

To separate the rare earth elements in quantity, it is necessary to use the chromatographic technique of displacement development. This requires large column loadings, in contrast to the trace loadings used for fission product analysis. Two methods have been proposed for establishing development conditions, using aminocarboxylic acids as the complexing agent. Both use two separately loaded ion-exchange columns, which are coupled together in series before elution. The rare earth mixture is loaded to the first column. FITCH and LORIERS both used a second column in the NH_4^+ form[7–9], while SPEDDING used metal-loaded columns (*e.g.* Fe^{3+}, Cu^{2+})[10,11].

The method used in the present work is similar to the first of the procedures described above, with, however, significant modifications. If ion-exchange columns loaded with Ln^{3+} ions are eluted with solutions of the ammonium salts of nitrilotriacetic acid (TRILO) or ethylenediaminetetraacetic acid (EDTA), lanthanons are eluted from the resin as complexes $Ln(LnA_2)$ for TRILO, and $Ln(LnY)_3$ for EDTA, where A = TRILO and Y = EDTA (Table I, columns 4 and 5).

The separation obtained under these conditions was poor. Fig. 1 shows an elution curve for the light earth material didymium oxide. This is due to a part of the lanthanon being eluted in cationic form as a counter-ion to the complex anions. It is known that lanthanon cations have similar affinities for ion-exchange materials (see refs. 1, 2 and Table VII).

This difficulty was overcome by using a column loaded with a controlled mixture of ammonium and lanthanon ions. Varying loadings were applied to ion-exchange columns by using the relative affinity data in Table VII, (see experimental section).

* Now at Thames Board Mills Ltd.

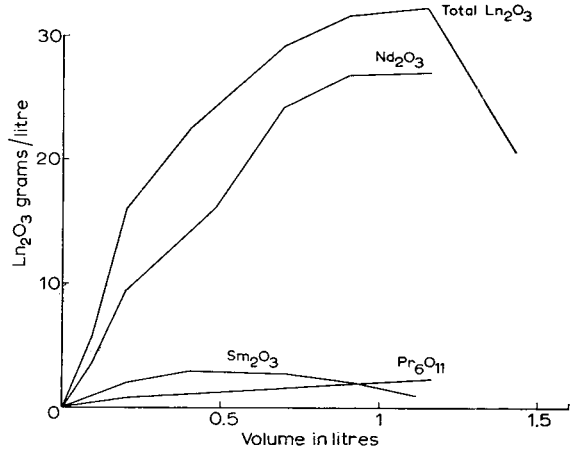


Fig. 1. Elution of 100% lanthanon loaded column. Eluant = 0.2 *M* TRILO in 0.5 *N* NH_3.

The ammonium ion does not form complexes with TRILO or EDTA, hence ammonium salts of the corresponding anionic complexes were formed on elution. The pH of the eluate depended on the lanthanon loading of the cation-exchange column, and, with TRILO, some control could be obtained by varying the eluant ammonium ion concentration. This was not possible with EDTA solutions, where use of less than the stoichiometric quantity of base caused blocking of the column by a precipitate of the acid. Some typical results are shown in Table II.

In all cases, visual indication of separation was obtained, and an elution curve is shown in Fig. 2. This differed considerably from the first case, and is due to the formation of anionic complexes under these conditions. When heavy earth–ammonium ion mixtures were eluted in the same manner, the anionic complexes formed had pH values from 0.2 to 0.4 units lower than in corresponding experiments with the

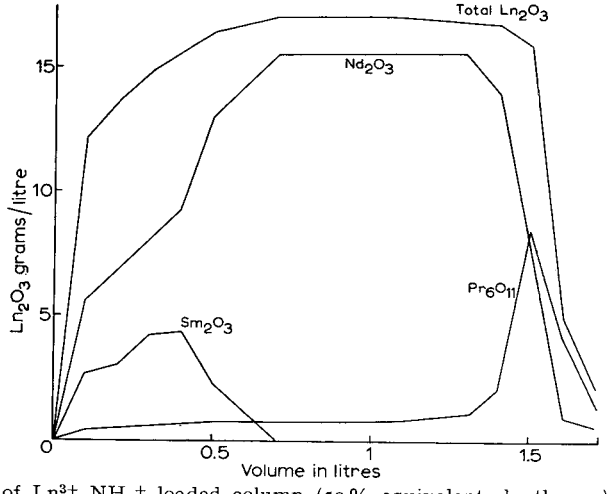


Fig. 2. Elution of Ln^{3+}–NH_4^+ loaded column (50% equivalents lanthanon). Eluant = 0.2 *M* TRILO in 0.5 *N* NH_3.

TABLE I

ELUTION OF LANTHANON-LOADED CATION EXCHANGE COLUMNS WITH COMPLEXING AGENT SOLUTIONS

Reagent	*Eluant concentration (moles/l)*	*Eluant (NH_4^+) (moles/l)*	*Eluate Ln_2O_3 (moles/l)*	*Calculated Ln_2O_3 (moles/l)*	*Eluate pH*
TRILO	0.2	0.5	0.193	0.20	2.03
	0.1	0.25	0.093	0.10	2.12
	0.1	0.3	0.108	0.10	3.90
EDTA	0.1	0.4	0.133	0.133	2.40

TABLE II

ELUTION OF Ln^{3+}–NH_4^+ LOADED CATION EXCHANGE COLUMNS WITH COMPLEXING AGENT SOLUTIONS

Reagent	*Lanthanon loading (% equiv.)*	*Eluant concentration (moles/l)*	*Eluant (NH_4^+) (moles/l)*	*Eluate pH*	*Eluate (NH_4^+) (moles/l)*	*Eluate Ln_2O_3 (moles/l)*
TRILO	80	0.2	0.5	2.45	0.134	0.130
	54	0.2	0.5	2.65	0.197	0.107
	38	0.2	0.5	2.80	—	0.094
	54	0.2	0.58	2.90	0.193	0.118
	54	0.2	0.4	2.50	—	0.084
	54	0.1	0.25	2.80	—	0.046
EDTA	54	0.1	0.4	3.0	0.101	0.100

TABLE III

ELUTION OF Ln^{3+}–NH_4^+ (HEAVY EARTH FRACTIONS) WITH 0.1 *M* EDTA SOLUTIONS

Fraction No.	*Yttrium oxide*					*Xenotime*				
	Yb_2O_3 %	*Tm_2O_3 %*	*Er_2O_3 %*	*Ho_2O_3 %*	*Dy_2O_3 %*	*Yb_2O_3 %*	*Tm_2O_3 %*	*Er_2O_3 %*	*Ho_2O_3 %*	*Dy_2O_3 %*
1	3.5	1.5	18	4	20	91	5.6	7.7	—	—
2	0.1	0.1	5	4	20	85	8.2	18.7	tr	3.1
3			0.1	1.5	30	21.5	3.5	33.4	—	4.7
4					35	3	—	4.2		7

light earths. This is due to the formation of stronger complexes between EDTA and the heavy earths. Results obtained by eluting two heavy earth fractions with EDTA solutions are shown in Table III; each eluate was divided into ten equal fractions, and the analysis of the first four is given.

Both of the heavy earth fractions contained a large proportion of yttrium. In each case the heavy earth elements as far as holmium were concentrated with the first third of the eluate.

The lanthanon anionic complexes formed on the first column have a low pH, which varied with the lanthanum–ammonium loading of the column. The NH_4^+/H^+ ratio of these solutions must be determined experimentally, since the relative affinity of the Ln^{3+}–NH_4^+ ions for the resin does not obey a mass action law. For optimum conditions, it is necessary to condition the development column to the same NH_4^+/H^+ ratio; there is also a lower limit, since if the H^+ loading of the development column is too great the complexing agent is precipitated. Conditions for loading the rare earth column and conditioning the development column in one operation are described in the experimental section.

When the lanthanon complexes pass to the development column, cationic lanthanon is exchanged to the resin phase. At the same pH, weak complexes have a greater cation content than stronger complexes, which tend to the solution phase and are eluted first from the column. The pH of the eluate increased in the development column, owing to the exchange of lanthanon cations for ammonium ions, and the lanthanon content decreased.

When elution was carried out with the stoichiometric salts of TRILO or EDTA, poor separations were obtained and the eluates had pH values from 1 to 2 units greater than those shown in Table II. This difficulty was overcome by buffering the eluant solutions. With TRILO, control was effected by adjusting the NH_4^+:com-

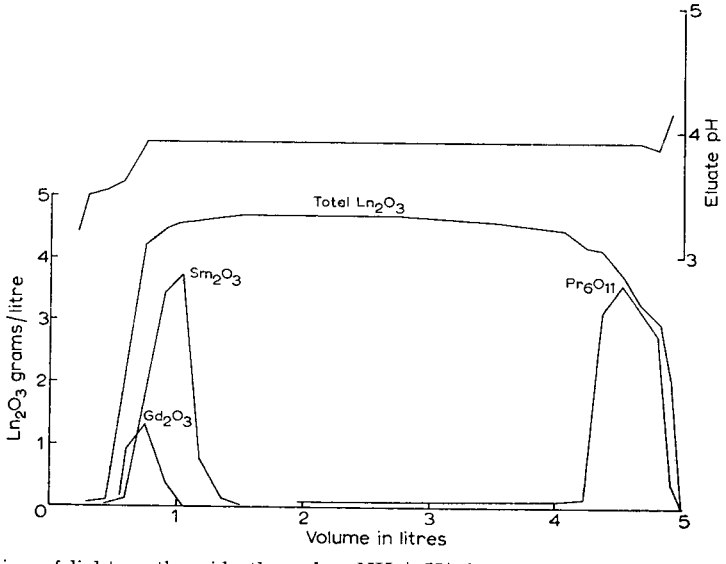


Fig. 3. Elution of light earth oxide through a NH_4^+–H^+ form development column. Eluant = 0.1 *M* TRILO in 0.25 *N* NH_3.

TABLE IV

SEPARATION OF XENOTIME FRACTION WITH 0.05 *M* EDTA + 0.1 *M* HCOOH

Fraction No.	Yb_2O_3 %	TmO_3 %	Er_2O_3 %	Ho_2O_3 %	Dy_2O_2 %	Sm_2O_3 %
1	80.5	5.8	13.8	—	1	
2	58	7.2	26	Tr	5	
3	42.7	14	20	Tr	14.5	
4	4	2	2	—	16	
5					2	
6					0.5	
7					—	1

plexing agent ratio, while formic acid (p*K* 3.75) was added to the EDTA solutions. The increase in pH of the eluate was then limited to 0.5 unit, and light earth separations of the type shown in Fig. 3 were obtained.

Elution of the rare earth elements rapidly followed "break-through" of the eluate. The major component (Nd_2O_3) was > 99 % pure, and the lanthanon content of the eluate half that required for a stoicheiometric anionic complex. A second elution of the neodymium fraction by the same technique gave a > 99.9 % product.

The method was less successful when applied to heavy earth materials. The results obtained by eluting a xenotime fraction with a buffered EDTA solution are shown in Table IV. Satisfactory control of the eluate pH was obtained, but the lanthanon content was 50 % greater than that observed in Fig. 3.

The distribution of lanthanon cations on the development column was examined with a sectioned column apparatus. When the light earth material was eluted, the resin in the development column contained about 5 % equivalents of lanthanon cations; with the heavy earth material, the lanthanon content varied from 2 % to 3 %.

DISCUSSION

The addition of controlled quantities of NH_4^+ ions to lanthanons on an ion-exchange column forces the rare earths to the anionic form on elution with a complexing agent solution. The partial separations obtained are due to the net reaction $Ln_1Y^{1-} + \overline{Ln_2}^{3+} \rightarrow Ln_2Y^{1-} + \overline{Ln_1}^{3+}$. The concentration of free lanthanon cations under these conditions is very small, so the separations obtained are greater than those obtained with a complete lanthanon loading. The lanthanon band is incompletely developed, and no quantitative estimate of the efficiency of the separation can be made. However, the method is very useful for making preliminary group separations of complex lanthanon mixtures, and in concentrating the less abundant elements[12].

The elution of the light earth elements on an NH_4^+–H^+ column with buffered eluates gave results that can be examined quantitatively. The "didymium oxide" contained 74 % Nd_2O_3, while the major fraction of the eluate contained > 99 % Nd_2O_3. (Fig. 3). This gave a 33-fold enrichment, which, taking the Nd-Pr separation factor as 2.5, gave 10 theoretical stages[13]. The equivalent plate height was thus 6 cm; in theory, this should approach the particle size of the resin, but such conditions are rarely approached even in elution experiments.

During elution, the amount of cationic lanthanon found on the development

column was small. A study of the distribution of lanthanon cations between the resin and solutions of rare earth anionic complexes showed the lanthanon content of the resin to vary inversely with solution concentration and pH. Table V shows the equilibrium lanthanon content of resin samples treated with TRILO and EDTA complexes of three lanthanons, adjusted to pH 3.0, the lanthanon content of each solution being 0.05*M*.

The lanthanon content of the resin decreased from neodymium to erbium, owing to the increased stability of the complexes formed by both reagents in the sequence Nd, Y, Er[13,14]. They are considerably greater than the amount of lanthanon found in the development column in elution experiments. However, a large excess of reagent was needed to obtain an equilibrium value, and this quantity increased with the stability of the complex. This is due to two effects, the low lanthanon

TABLE V

EQUILIBRIUM LANTHANON CONTENT OF RESIN AFTER TREATMENT WITH LANTHANON COMPLEXES

Lanthanon	*Lanthanon content of resin (% equiv.)*	
	TRILO complex	*EDTA complex*
Nd	55	79
Y	42	56
Er	27	39

cation content of the solutions, and the increase in pH observed when these are exchanged for ammonium ions. Hence smaller quantities of heavy earths are exchanged on the development column, resulting in poor separations.

However, the experimental method described is very convenient for separating the light earth elements, since it is rapid and no metal–rare earth separations are involved. Some advantage would be expected from the use of more dilute eluate solutions, at the expense of prolonging the separation. An examination of weaker complexing agents, which would give an increased reflux on the development column, merits further study.

EXPERIMENTAL

Materials

Three rare earth materials were used in this work. Two of these, "didymium oxide" and "yttrium oxide" were purchased from Thorium Ltd. The third material, xenotime, was extracted from the mineral after fusion with caustic soda. Analysis of these materials is given in Table VI.

TABLE VI

ANALYSIS OF RARE EARTH MATERIALS

Material	CeO_2	Pr_6O_{11}	Nd_2O_3	Sm_2O_3	Gd_2O_3	Tb_4O_7	Dy_2O_3	Ho_2O_3	Er_2O_3	Tm_2O_3	Yb_2O_3	Lu_2O_3	Y_2O_3
"Didymium"		10.5	74	9.5	6								
"Yttrium"					6.9	1.2	11.9	3.4	6.9	0.3	2.3	0.1	65
Xenotime	0.6	0.1	0.5	1.0	2	1	14.5	2.5	12.5	2	17.2	3	45

Analytical methods for lanthanons

Gravimetric methods were used for the isolation of lanthanon fractions. An excess of oxalic acid was added to the lanthanon solution, the pH adjusted to 2.0, and the precipitate coarsened by heating for 1–2 h. After cooling and filtering the precipitate was ignited in platinum at 800–900° to constant weight. Tests on light and heavy lanthanon fractions showed the recovery to be > 99.9%. If complexing agents were present, the pH of the solution was adjusted to 1.5 and the solution allowed to stand overnight before filtering off the precipitated reagent; lanthanons were determined in the filtrate.

The concentration of lanthanons was determined with a spectrophotometer (Beckman D.U.). Calibration curves were prepared from chloride solutions of "Specpure" materials (Johnson Matthey and Co.). Heavy earth fractions from the low-grade "yttrium oxide" were analysed spectrographically using the "iron-flux" method[15].

Complexing agents

EDTA and TRILO were purchased from the Geigy Company and Norman Evans and Rais. The acids were purified by solution in ammonia, treatment with charcoal, and reprecipitation.

The concentration of complexing agent solutions was determined by a modification of SIGGIA'S method[16]. An aliquot of the solution was boiled with caustic soda to expel ammonia, and pyridine (25 % v/v) was added. After adjusting the pH to 7 with hydrochloric acid, the solution was titrated potentiometrically with a standard copper solution, using a platinum–calomel electrode system. The end-point was indicated by an abrupt change in potential of the order of 100–200 mV.

Relative affinity determinations

A modification of REICHENBERG'S method was used[17]. Samples (10 ml) of resin were enclosed between two small sinter tubes (porosity 2) joined with a section of P.V.C. tubing. The total exchange capacity of the resin was determined by H^+ displacement. One column was converted to the NH_4^+ form and a second to the Ln^{3+} form. Each column was treated with a 10-fold excess of conditioning solution, containing a known

TABLE VII

RELATIVE AFFINITY OF AMMONIUM AND LANTHANON IONS FOR THE RESIN ZEO-KARB 225

Lanthanon fraction	*Solution concentration ($Ln^{3+} + NH_4$) (equiv./l)*	*Lanthanon concentration (equiv./l)*	*Lanthanon loading of resin (% equiv.)*
"Didymium"	1	0.25	66.3
		0.15	55.5
		0.10	53.3
	2	0.8	71.7
		0.6	63.7
		0.4	49.7
"Yttrium"	1	0.075	46.0
		0.050	45.2
	2	0.4	48.7

ratio of ($Ln^{3+} + NH_4^+$) ions. The columns were drained at the pump, and rapidly water-washed to remove excess conditioning solution. They were stripped quantitatively with a potassium–EDTA solution, and ammonia and lanthanon determinations made on the filtrate; in the latter case, two oxalate precipitations were given to minimise potassium occlusion. The ($Ln^{3+} + NH_4^+$) content of each column agreed well with the capacity determined by hydrogen displacement, and the ratios in either case were identical, showing that equilibrium had been attained.

Results obtained with two of the lanthanon fractions described in Table VI are summarised in Table VII.

Little difference in affinity was found between the "light" and "heavy" earths. The effect of changing the ionic concentration was qualitatively in agreement with theory, but the results did not fit a mass action law.

Ion-exchange technique

The cation-exchange resin Zeo-Karb 225 (250 μ fraction) was used. It was back-washed to remove fines, and traces of heavy metal were removed by treating the resin, in the ammonium form, with a complexing agent solution.

The elution experiments described in Tables I–III were carried out in columns 60 cm long by 8.5 cm^2. These were conditioned with an excess of reagents and washed with water before elution. Separation experiments (Fig. 3 and Table IV) were carried out in columns 120 cm long by 8.5 cm^2. To carry out the loading and conditioning operations together, use was made of the affinity data of Table VII and data obtained by the elution of Ln^{3+}–NH_4^+ columns (Table II). Solution concentrations were selected to give a 50 % equivalent lanthanon loading to the first half of the column, and the pH was adjusted to give a suitable NH_4^+:H^+ ratio. Convenient solution concentrations were 0.1 *N* Ln^{3+} + 0.9 *N* NH_4^+ at pH 1.7 or 0.4 *N* Ln^{3+} + 1.6 *N* NH_4^+ at pH 1.4. The eluate was sampled with a variable volume fraction collector using a flow rate of 0.5 cm/min.

Distribution of lanthanons on the resin column was examined with a "sectioned column" apparatus. Eight 30 cm × 8.5 cm^2 columns were coupled together in series, each column being provided with a 3-way tap for drainage. The columns were loaded and washed in the usual way. Elution was carried out until the complexing agent solution was detected in the eluate. The individual columns were then disconnected, drained at the pump, and the distribution of lanthanon between the two phases was determined.

Preparation of lanthanon anionic complexes

MARSH'S method was used for the preparation of a number of lanthanon anionic complexes[18]. The complexing agent and a small excess of lanthanon oxide (1–2 %) was refluxed with stirring, and ammonia added dropwise. After heating for 1–2 h a small residue of lanthanon oxide remained undissolved. This was filtered off after boiling off the excess ammonia. Neutral solutions were obtained, analysis agreeing with the composition NH_4LnY for the EDTA complexes, and $(NH_4)_2H\ LnA_2$ for TRILO complexes.

The data of Table V were obtained by the technique used for determining the affinities of Ln^{3+}:NH_4^+ ions. In these cases, the conditioning solutions were lanthanon anionic complexes.

ACKNOWLEDGEMENTS

We would like to thank F. H. HOWIE and D. SWEASEY for experimental work, and J. WALKDEN (U.K.A.E.A.) for much spectrographic work. Valuable discussions with Dr. J. LORIERS and Dr. J. K. AIKEN are acknowledged. This work formed part of the programme of the National Chemical Laboratory and is published by permission of the Director.

SUMMARY

Methods for separating the rare earth elements by ion-exchange chromatography are discussed.

The elution of cation-exchange columns, completely loaded with rare earth ions, with complexing agent solutions gave eluates containing cationic and anionic lanthanon. However, if the ion-exchange column was loaded with a controlled Ln^{3+}–NH_4^+ mixture, elution gave predominantly an anionic lanthanon complex. Partial separations are obtained under these conditions, and this was shown to be a useful method for the preliminary concentration of complex lanthanon mixtures.

Conditions for separating the light earths, using an NH_4^+–H^+ form development column, were established. This method was not successful for separating the heavy earths, and the reasons for this are discussed.

REFERENCES

1 R. G. RUSSELL AND D. W. PEARCE, *J. Am. Chem. Soc.*, 65 (1943) 595.
2 B. A. LISTER AND M. L. SMITH, *J. Chem. Soc.*, (1948) 1272.
3 D. ERAMATSA, G. SAHAMA AND V. KANULA, *Ann. Acad. Sci. Fennicae*, A 57 (1941) 5.
4 B. H. KETELLE AND G. E. BOYD, *J. Am. Chem. Soc.*, 69 (1947) 2800.
5 D. H. HARRIS AND E. R. TOMPKINS, *J. Am. Chem. Soc.*, 69 (1947) 2792.
6 A. E. MARTELL AND M. CALVIN, *Chemistry of the Metal Chelate Compounds*, Prentice Hall, New York, 1952, Chap. 9.
7 F. T. FITCH AND D. S. RUSSELL, *Can. J. Chem.*, 29 (1951) 363.
8 J. LORIERS AND D. CARMINATI, *Compt. Rend.*, 237 (1953) 1328.
9 J. LORIERS AND J. QUESNEY, *Compt. Rend.*, 239 (1954) 1643.
10 F. H. SPEDDING, J. E. POWELL AND E. J. WHEELWRIGHT, *J. Am. Chem. Soc.*, 76 (1954) 612.
11 F. H. SPEDDING, J. E. POWELL AND E. J. WHEELWRIGHT, *J. Am. Chem. Soc.*, 76 (1954) 2557.
12 *Brit. Pat.*, 880,561 to N. E. TOPP AND D. D. YOUNG, 1961.
13 G. ANDEREGG, *Helv. Chim. Acta*, 43 (1960) 825.
14 G. SCHWARZENBACH, R. GUT AND G. ANDEREGG, *Helv. Chim. Acta*, 37 (1954) 937.
15 Springfields Chemicals Services, Method No. 357 (1954).
16 S. SIGGIA, D. W. EICHLIN AND D. RHEINHART, *Anal. Chem.*, 27 (1955) 1745.
17 D. REICHENBERG, K. W. PEPPER AND D. J. MCCAULEY, *J. Chem. Soc.*, (1951) 493.
18 J. K. MARSH, *J. Chem. Soc.*, (1951) 3057.

ION EXCHANGE PROCEDURES

IV. SEPARATION OF ^{234}Th (UX_1) FROM URANYL NITRATE SOLUTIONS*,**

TAKEO MURASE***, EDMUND L. LIND§ AND FREDERICK NELSON

Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. (U.S.A.)

(Received November 10th, 1963)

In studies involving various equilibria and reactions of Th(IV), use of a thorium tracer is sometimes desirable to facilitate analyses. The tracer ^{230}Th (ionium), $T_{1/2} = 8 \cdot 10^4$ years, is often selected for this purpose, but its analysis requires α-counting which is time consuming. A more convenient tracer is ^{234}Th (UX_1), $T_{1/2} = 24.1$ days. Being a γ-emitter, it may readily be determined in liquid samples by scintillation counting. The isotope is also readily available since it arises from decay of uranium (^{238}U).

The procedure described here was developed for routinely separating ^{234}Th from uranium and other activities associated with uranium; it is a modification of the cation exchange method first used by BANE[2] and DYRSSEN[3].

DISCUSSION

The procedure was developed specifically for recovering ^{234}Th from uranyl nitrate hexahydrate, selected because this compound is readily available and very soluble. The procedure was designed for processing *ca.* 400 g of this salt. This contains 63 μC of ^{234}Th at secular equilibrium which is sufficient for a considerable amount of tracer work. If desired, the procedure can readily be adapted to handling substantially larger amounts of uranium.

The method is based on the very high selectivity of cation exchangers of the Dowex 50 type for Th(IV). The salt is dissolved in sufficient 0.1 *M* HNO_3 to yield a 0.4 *M* U(VI) solution. This solution is passed into a small bed of cation exchange resin (*ca.* 10 ml bed volume) to concentrate Th on the resin bed. During this step, the bed is converted essentially completely to the UO_2^{2+} form; however, the adsorbed uranium is readily eluted by 6 *M* HCl while leaving Th(IV) on the resin.

Th(IV) is removed from the resin in a sharp band with 6 *M* HCl–1 *M* HF solution, presumably through formation of weakly adsorbed fluoride complexes. Although sulfuric acid or organic complexing agents, *e.g.*, citrates, oxalates, etc., may also be

---

* This document is based on work performed at the Oak Ridge National Laboratory, Oak Ridge, Tenn., operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

** For Part III, see ref. 1.

*** Guest scientist at Oak Ridge National Laboratory 1961/1962, present address: Atomic Fuel Corporation, Tokyo, Japan.

§ Oak Ridge Institute of Nuclear Studies Research Participant at ORNL, 1962–1963, present address: Central Washington State College, Ellensburg, Washington.

used to elute Th(IV) from cation exchangers[4], use of HCl–HF media is preferred since these acids are readily removed by evaporation in subsequent concentration steps.

The thorium fraction usually still contains small amounts of uranium, Fe(III) and other impurities, such as resin degradation products including traces of sulfate, so that further purification is desirable. Organic matter is destroyed by evaporation to near-dryness with concentrated $HClO_4$–HNO_3 solution. The residue is dissolved in a small amount of 6 *M* HCl and added to a second (small) cation exchange column. A fine mesh resin of relatively low cross-linking (4 %) is used in the second purification step to improve rates of elution. This resin should be of "analytical grade". This second column is washed sequentially with 6 *M* HCl and 4 *M* HCl to remove U(VI) and Fe(III) impurities; Th(IV) is then eluted with 6 *M* HCl–1 *M* HF.

The uranium nitrate solution can be stored; after an appropriate time for growth of the ^{234}Th daughter, it can be reprocessed.

This method of isolation of UX_1 is relatively slow since about 20 h are required for the concentration step. However, we consider it preferable to a more rapid carrier technique (*e.g.* co-precipitation with LaF_3) since fewer manipulations are required; the column can be operated essentially without attention during the concentration step. A relatively slow flow rate is also recommended for the purification step, since in this way high product purity is assured.

TYPICAL SEPARATION

About 400 g of uranyl nitrate hexahydrate were dissolved in 2 l of 0.1 *M* HNO_3 and the solution passed into a 1.75 cm^2 × 5.7 cm (10 ml) column of water-washed Dowex 50-X8 resin (100–200 mesh) in the hydrogen form. Flow rate was adjusted to about 1 cm/min. After the solution had passed through, it was washed with 4 column volumes (c.v.) of 6 *M* HCl and then with 4 c.v. of 6 *M* HCl–1 *M* HF to elute the thorium (UX_1). The eluent containing thorium was collected directly in a Teflon evaporating dish, 1 ml each of concentrated $HClO_4$ and HNO_3 were added and the solution evaporated to a small volume (0.1 ml). The sample was taken up in 1 ml of 6 *M* HCl and added to a 0.28 cm^2 × 3 cm column of Dowex 50-X4 resin (200–400 mesh) which had been pretreated with 6 *M* HCl. The column was washed with 1 c.v. of 6 *M* HCl and then with 9 c.v. of 4 *M* HCl. Fig. 1 gives a plot of counting rate *versus* column volumes of effluent. The elution band which appears with maximum counting rate near 2.5 c.v. was found by decay rate measurements and γ-spectroscopy to consists primarily of 1.18 min ^{234}Pa (UX_2) and 6.7 h ^{234}Pa (UZ), arising from decay of ^{234}Th. Thorium was removed in a sharp band with 6 *M* HCl–1 *M* HF solutin.

PROCEDURE

(*a*) *Materials and reagents*

Resins. Dowex 50W-X8, 100–200 mesh, hydrogen-form cation exchanger. Dowex 50W-X4, 200–400 mesh, "analytical grade" hydrogen-form cation exchanger.

Apparatus. Two sizes of polyethylene tubing, 1.5 cm diameter (area 1.75 cm^2) and 0.6 cm diameter (area 0.28 cm^2) in 10 cm lengths are used to prepare columns. A one-hole rubber stopper, carrying a small section of plastic tubing containing a porous Teflon plug, is inserted in the larger tube to retain resin. The smaller tube (used for the second column) is softened at one end by heating, pulled to a tip and a

porous Teflon plug is inserted. Additional apparatus includes 2 l glass containers plastic test tubes, Teflon evaporating dishes, plastic transfer pipets, and syringes.

Columns. The columns used are given in Table I.

Solutions. 6 *M* HCl; 4 *M* HCl; 6 *M* HCl–1 *M* HF; conc. $HClO_4$; conc. HNO_3.

TABLE I

	Column 1 Concentration step	*Column 2 Purification step*
Resin	Dowex 50	Dowex 50
Cross-linking	X8	X4
Form	H^+	H^+
Mesh size	100–200	200–400
Column length (cm)	5.7	3
Flow rate (cm/min)	1	0.4
Temp. (°C)	25	25
Load solution	0.4 *M* $UO_2(NO_3)_2$–0.1 *M* HNO_3	6 *M* HCl
Load solution volume	2 l	1 ml
Wash solution and volume (column volumes, c.v.)	6 *M* HCl (6 c.v.)	(1) 6 *M* HCl (1 c.v.) (2) 4 *M* HCl (9 c.v.)
Eluent for UX_1 fraction and volume (c.v.)	6 *M* HCl–1 *M* HF (6 c.v.)	6 *M* HCl–1 *M* HF (6 c.v.)

(*b*) *Feed preparations*

400 g of uranyl nitrate hexahydrate are dissolved in 2 l of water containing 14 ml of concentrated HNO_3. Final composition is about 0.1 *M* HNO_3–0.4 *M* $UO_2(NO_3)_2$.

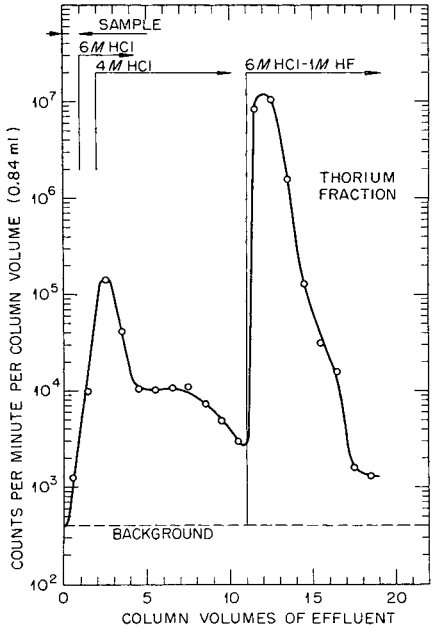


Fig. 1. Counting rate of effluent fractions in purification of Th(IV) by cation exchange (Dowex 50-X4, 200–400 mesh, 3 cm × 0.28 cm^2 column, 25°).

(c) *Column operations and effluent treatment*

1. *Concentration of* ^{234}Th. Resin as a slurry in water is added to the larger column to form a bed about 6 cm in length. Uranyl nitrate solution is introduced through tubing connected to the top of the resin column by a one-hole rubber stopper. The reservoir of feed solution is placed at a sufficient height (about 20 in.) above the resin bed to produce the desired flow rate. About 20 h are required for the concentration step.

After the feed has passed through the resin bed, the bed is washed with 4 c.v. of 6 *M* HCl to elute excess U(VI); Th(IV) is eluted with 4 c.v. of 6 *M* HCl–1 *M* HF.

2. *Purification of* ^{234}Th. The HCl–HF effluent is collected in a Teflon dish; 1 ml each of concentrated $HClO_4$ and concentrated HNO_3 are added and the solution is evaporated to a small drop of about 0.1 ml. This is taken up in 1 ml of warm 6 *M* HCl to yield a Th(IV) concentrate of about 1 c.v. for the next operation.

Dowex 50W-X4 "analytical grade" resin as a slurry in water is added to the smaller plastic column until a bed about 3 cm in length is formed. The column is pretreated with 6 *M* HCl and the ^{234}Th concentrate is added. The column is operated at a flow rate of 0.4 cm/min. When the sample has passed into the resin bed, 1 c.v. of 6 *M* HCl is added as rinse. This is followed by 9 c.v. of 4 *M* HCl. The Th (IV) is eluted into a Teflon dish with 5 ml (6 c.v.) of 6 *M* HCl–1 *M* HF. This effluent may be reduced in volume, and the HF driven off by fuming with concentrated HNO_3 or $HClO_4$. About 2 h are required for the final purification step.

ACKNOWLEDGEMENT

The authors are indebted to Dr. K. A. KRAUS for valuable comments and discussion.

SUMMARY

A cation exchange procedure is described for recovering microcurie amounts of carrier-free UX_1 (^{234}Th) from uranyl nitrate solution. Two ion exchange columns are employed, one for initial concentration and the other for final purification.

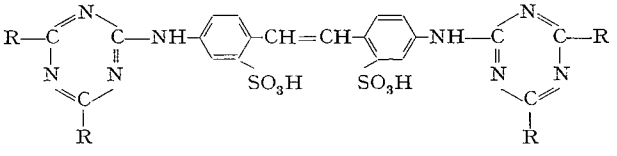
REFERENCES

1 F. NELSON, D. C. MICHELSON AND J. H. HOLLOWAY, *J. Chromatog.*, 14 (1964) 258.
2 R. W. BANE, *Rept.* CC-3336, Nov. 23 (1945).
3 D. DYRSSEN, *Svensk Kem. Tidskr.*, 62 (1950) 153.
4 E. K. HYDE, *Proc. Intern. Conf. Peaceful Uses At. Energy, Geneva, 1955* (United Nations, 1956), Vol. VII, p. 281.

Short Communications

Chromatographie der optischen Aufhellmittel auf Basis der 4,4'-Diaminostilben-2,2'-disulfosäure

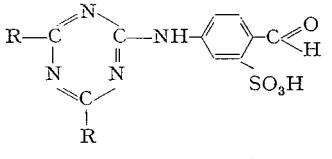
Beim Ausarbeiten der chromatographischen Methodik zur Auftrennung, Identifizierung und Betriebsanalyse der optischen Aufhellmittel auf der Basis der 4,4'-Diaminostilben-2,2'-disulfosäure und Cyanurchlorid (I) waren wir mit den Resultaten der Papierchromatographie nicht zufrieden. Bei der Trennung auf dem Papier waren die Flecken der Substantivität wegen langgezogen und die Unterschiede zwischen den getrennten Stoffen nur schwierig wahrnehmbar.



(R= —OH, $-NH_2$, $-OCH_3$, —Cl, $-NHC_6H_5$)

(I)

Um diese Schwierigkeiten zu beseitigen und die Trennung zu verbessern versuchten wir von prinzipiell zwei Methoden Gebrauch zu machen: Die erste Methode, bei der wir eine solche Art der Chromatographie suchten, bei der die Substantivität nicht zum Ausdruck kommen könnte, konnten wir durch Anwendung der Dünnschichten von Kieselguhr realisieren. Die Substantivität dieser Stoffe (I) verursacht bei Anwendung derselben Lösungsmittelsysteme wie bei der Papierchromatographie keine Schwierigkeiten — die Flecken sind gut abgetrennt und scharf begrenzt. Die zweite Methode bestand in der Überführung der zu trennenden Stoffe in solche Produkte, die die Substantivität nicht besitzen. Wir wandten hier die Oxydation der Stilbendoppelbindung mittels Kaliumpermanganat an[1], bei der zwei Moleküle der entsprechenden Aldehyde (II) gebildet werden.



(R= —OH, $-NH_2$, $-OCH_3$, —Cl, $-NHC_6H_5$)

(II)

Diese Verbindungen weisen eine Reihe von guten Eigenschaften auf, die bei der Identifizierung ausgenützt werden können.

Die Auftrennung der ursprünglichen Verbindungen I sowie ihrer Oxydationsprodukte wird hauptsächlich von den Substituenten auf dem Triazinring beeinflusst. Die kleinsten R_F-Werte haben diejenigen Produkte, wo R die –OH Gruppen sind. Die R_F-Werte steigen dann über die NH_2-Derivate zu CH_3O- und bis zu C_6H_5NH-Derivaten, welche die höchsten R_F-Werte zeigen. Es hat sich gezeigt, dass in geeigneten Lösungsmittelsystemen auch die Produkte mit den reaktiven Cl-Atomen auf dem Triazinring chromatographiert werden können.

Mittels der Papierchromatographie können bei den Verbindungen des I-Typs die *trans*- und *cis*-Formen unterschieden werden. Die *cis*-Derivate, deren Moleküle nicht planar sind und die keinen substantiven Charakter haben[2], bilden normale runde Flecken, die stets höhere R_F-Werte besitzen, als die entsprechenden, stark langgezogenen Streifen der *trans*-Derivate. Bei der Dünnschichtchromatographie ist die Folge der *cis*- und *trans*-Derivate umgekehrt. Die *cis*-Isomere haben kleinere R_F-Werte als die *trans*-Isomere. Wir beobachteten, dass bei der Oxydation mit Kaliumpermanganat die *trans*-Isomere leichter oxydiert werden als die *cis*-Isomere. Nach der Oxydation liefern beide Formen dieselben Produkte. Die *cis*-Isomere können weiter von den *trans*-Isomeren mittels der Papierelektrophorese unterschieden werden. Die stark substantiven *trans*-Isomere wandern bei dieser Methode fast überhaupt nicht, die *cis*-Isomere verhalten sich normal. Die grösste Wanderungsgeschwindigkeit zeigen die *cis*-Derivate, bei denen R = –OH, und die kleinste, bei denen R = $-NHC_6H_5$.

Wir sind der Meinung, dass die Dünnschichtchromatographie auf Kieselgur auch für andere Klassen substantiver und stark polarer Verbindungen, besonders ausgesprochen substantiver Farbstoffe, anwendbar sein wird. Der oxydative Abbau der Stilbenderivate ist für diese charakteristisch, ist analog dem reduktiven Abbau der Azofarbstoffe[3] und zeigt einen anderen Weg, durch den die Schwierigkeiten mit der Papierchromatographie der Verbindungen I beseitigt werden können.

In unserem Laboratorium studieren wir weiter ausgehend die Papierchromatographie, die Dünnschichtchromatographie, sowie die Papierelektrophorese der Verbindungen I.

Experimenteller Teil

Es wurde auf dem Whatmanpapier Nr. 1 absteigend chromatographiert. Von den chromatographierten Stoffen wurden stets 2–3 μl der 1–2 %igen wässrigen Lösungen aufgetragen. Auf ähnliche Weise wurde auch auf den Dünnschichten von Kieselgel G nach Stahl (Merck) chromatographiert. Die Oxydationsprodukte II wurden für die Papierchromatographie in Form 2 %iger Lösungen wie folgt dargestellt: 0.1 g der Verbindungen I wurde in 3.5 ml 50 %igen wässrigen Pyridin aufgelöst und danach 1.5 ml ca. 2 *N* Kaliumpermanganat zugefügt, durchgeschüttelt und 15 Min bei Zimmertemperatur stehen gelassen. Dann wurde zu der Lösung eine kleine Menge festen $Na_2S_2O_3$ zugefügt, um den Überschuss an Kaliumpermanganat zu zerstören und durchgeschüttelt. Die entfärbte Lösung wurde durch Filtration von MnO_2 befreit und von dem Filtrat wurden auf das Chromatogramm 4 μl aufgetragen. Zum Entwickeln wurden folgende Lösungsmittelsysteme benutzt: *n*-Propanol–5 %ige wässrige $NaHCO_3$-Lösung (2:1)[4], *n*-Butanol–Pyridin–Wasser (1:1:1), *n*-Butanol–Pyridin–25 % Ammoniak (1:1:1) und Amylalkohol–Pyridin–25 % Ammoniak (1:1:1).

Die Sichtbarmachung der Verbindungen I geschah im Licht der U.V.-Lampe. Die

Spaltungsprodukte nach der $KMnO_4$ Oxydation wurden durch Besprühen mit einer 0.5 %iger Phenylhydrazinhydrochloridlösung sichtbar gemacht. Es werden Phenylhydrazone gebildet, die im U.V.-Licht blaugrün fluoreszieren.

*Východočeské Chemické Závody "Synthesia",** J. LATINÁK
Pardubice-Semtín (Tschechoslowakei)

[1] A. G. GREEN UND A. R. WAHL, *Ber.*, 30 (1897) 3097.
[2] H. THEIDEL, *Textil-Praxis*, 18 (1963) 773.
[3] J. PANCHÁRTEK, Z. J. ALLAN UND F. MUŽÍK, *Collection Czech. Chem. Commun.*, 25 (1960) 2783.
[4] J. LATINÁK UND L. SKALICKÝ, *Collection Czech. Chem. Commun.*, 22 (1957) 967.

Eingegangen den 24. Januar 1964

* Ostböhmische Chemische Werke "Synthesia".

Chromatographische Trennung substantiver Farbstoffe

Bei der Papierchromatographie der wasserlöslichen Azofarbstoffe verursacht die Affinität dieser Farbstoffe zur Zellulose — die Substantivität — grosse Schwierigkeiten. Die Farbstoffe werden oft sehr stark auf Zellulose adsorbiert, sodass sie am Chromatogramm nur sehr langsam wandern (das Entwickeln erfordert auch einige Tage) und überdies nicht gut entwickelte Flecken, sondern langgezogene Streifen liefern. Eine gewisse Substantivität gegenüber der Zellulose weisen auch einige einfache zu den sauren Wollfarbstoffen gehörende Farbstoffe, bei welchen diese Eigenschaft nicht erwartet wurde. Einige Autoren[1–3] waren bestrebt, die Bedingungen der Chromatographie dieser Farbstoffe derart zu modifizieren, dass ihre Ergebnisse wenigstens annehmbar sind. Sie wurden entweder von dem Bemühen geleitet das Zellulosepapier durch acetyliertes, Glasfaser- oder Aminoäthylzellulosepapier zu ersetzen, oder sie versuchten durch Änderung des Verfahrens oder Anwendung neuerer Lösungsmittelsysteme bessere Ergebnisse zu erzielen. Nachdem LATINÁK[4] zur Auftrennung der substantiven optischen Aufhellmittel mit grossem Erfolg die Dünnschichtchromatographie an Kieselgel zur Anwendung brachte, versuchten auch wir in unserem Laboratorium diese Methode zur Auftrennung der substantiven Azofarbstoffe anzuwenden, indem wir mit denselben Lösungsmittelsystemen wie bei der Papierchromatographie, d.i. *n*-Propanol–Ammoniak (2:1), Pyridin–*n*-Amylalkohol–Ammoniak (1:1:1) u.ä., arbeiteten. In der Fig. 1 sind die Photographien sowie der Dünnschichtchromatogramme an Kieselgel, als auch der Papierchromatogramme einiger substantiver Farbstoffe unter Anwendung der gleichen mobilen Phasen wiedergegeben. Aus der Abbildung ist ersichtlich, dass durch die Anwendung der Kieselgelschichten mit Wasser als stationäre Phase alle die genannten Schwierigkeiten bei der Papierchromatographie, die durch die Substantivität oder durch die Bindung mit Zellulose im Falle der Reaktivfarbstoffe verursacht werden, beseitigt werden. Unsere bisherigen Erfahrungen zeigen, dass durch richtige Wahl geeigneter mobiler Phasen Fall von Fall die gewünschten Trennungen erzielt werden können. In unserem

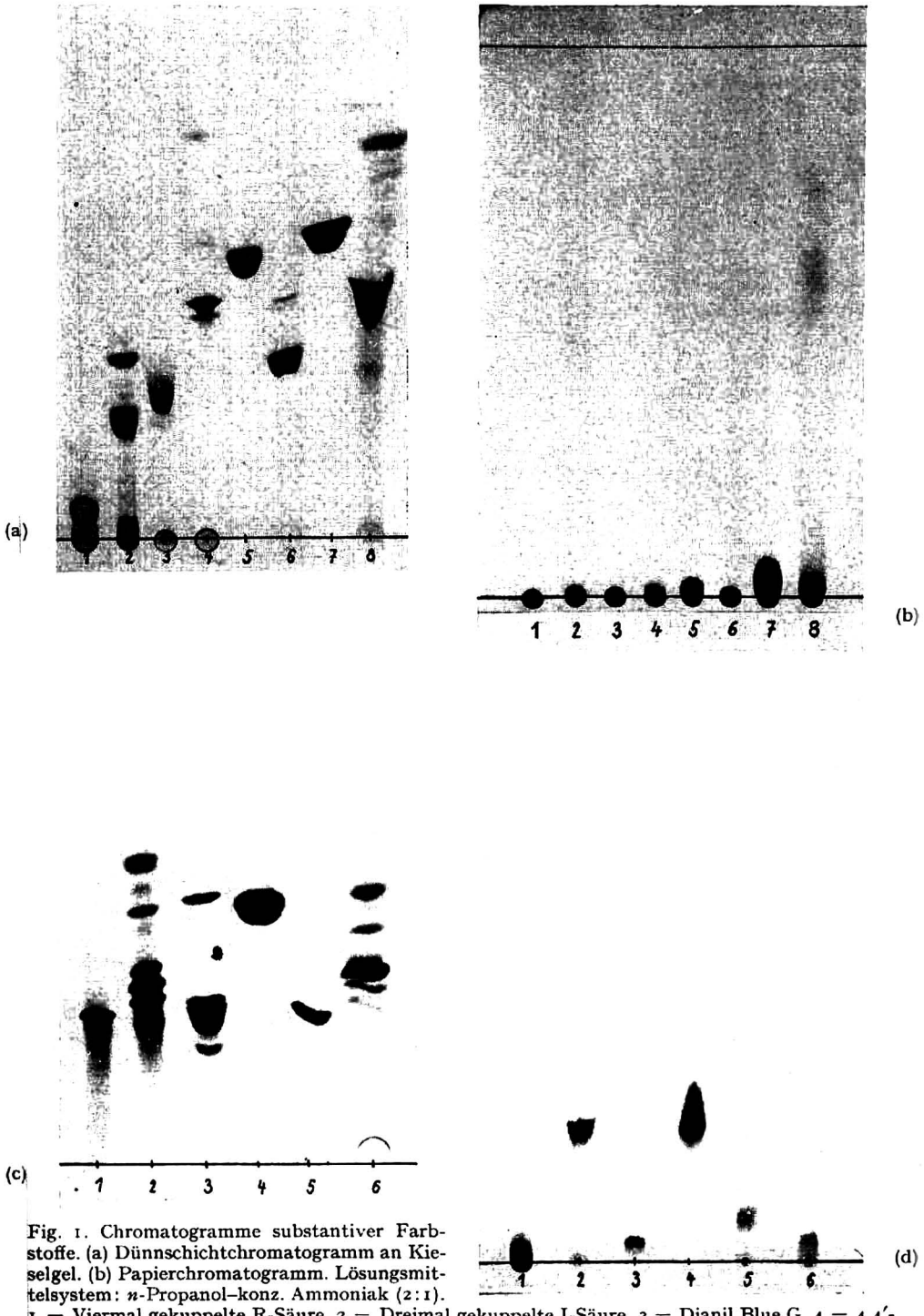


Fig. 1. Chromatogramme substantiver Farbstoffe. (a) Dünnschichtchromatogramm an Kieselgel. (b) Papierchromatogramm. Lösungsmittelsystem: *n*-Propanol–konz. Ammoniak (2:1). 1 = Viermal gekuppelte R-Säure. 2 = Dreimal gekuppelte I-Säure. 3 = Dianil Blue G. 4 = 4,4′-Diaminodiphenylsulphon → Naphthionsäure. 5 = Benzopurpurin 10B. 6 = Chicago Blue B. 7 = CongoRed. 8 = Sellaechtbraun DGR. (c) Dünnschichtchromatogramm an Kieselgel. (d) Papierchromatogramm. Lösungsmittelsystem: Pyridin–*n*-Amylalkohol–Ammoniak (1:1:1). 1 = Solophenylgrau 4G. 2 = Sellaechtbraun DGR. 3 = Diphenylechtgrün BE. 4 = Congo Red. 5 = Chicago Blue B. 6 = 4,4′-Diaminodiphenylsulphon → Naphthionsäure.

Laboratorium studieren wir systematisch die Papier- sowie Dünnschichtchromatographie sowohl verschiedener Handelstypen, als auch verschiedener gewählter Modellfarbstoffe und wollen in weiteren Mitteilungen über diese Resultate referieren.

Dünnschichtchromatographie

20 g Kieselgel G (Merck) wurde mit 40 ml Wasser angerührt und auf Platten (20 × 17 cm) in üblicher Weise verteilt. Die Platten wurden am nächsten Tag lufttrocken verwendet. Die zu chromatographieren Farbstoffe wurden in Form ca. 5 %iger wässriger Lösungen (5 μl) aufgetragen und das Entwickeln geschah aufsteigend in üblicher Weise unter Anwendung des Lösungsmittelsystems *n*-Propanol–Ammoniak (2:1), Pyridin–*n*-Amylalkohol–Ammoniak (1:1:1) u.ä.

Forschungsinstitut für Organische Synthesen,
Pardubice-Rybitví (Tschechoslowakei)

J. GASPARIČ
A. CEE

[1] L. MECKEL, *Textil-Rundschau*, 16, No. 7 (1961) 1.
[2] J. C. BROWN, *J. Soc. Dyers Colourists*, 76 (1960) 536.
[3] J. KOLŠEK, *Chemiker Ztg.*, 82 (1958) 35, 457; 83 (1959) 478.
[4] J. LATINÁK, *J. Chromatog.*, 14 (1964) 482.

Eingegangen den 24. Januar 1964

Gel filtration of lipid mixtures*

Gel filtration has proven to be a very useful technique for the fractionation of water-soluble materials but the technique has not been extended to the fractionation of lipids because the polysaccharide derivative generally used as a gel filtration medium does not swell in non-polar solvents. Beads of polystyrene crosslinked with small amounts of divinylbenzene swell extensively in benzene, suggesting the possibility of fractionating benzene-soluble mixtures on columns of these beads. VAUGHN[1] has reported the fractionation of polystyrene on similar columns.

Although simple and complex lipids do not differ greatly in molecular weight, phosphatides associate into micelles in non-polar solvents[2–6], while non-polar lipids, such as triglycerides, sterols and sterol esters apparently have little tendency to do so. Thus the basis exists for a gel filtration separation of lipid mixtures into relatively polar and non-polar fractions. The usual methods for carrying out this separation have been acetone precipitation of phosphatides or chromatography, usually on silicic acid columns[7]. Acetone precipitation often results in inclusion of saturated triglycerides and sterol esters in the "phosphatide" fraction and the galactosylglyceride lipids of plants are found in the acetone-soluble fraction[8]. Column chromatography is relatively time-consuming on a preparative scale and can result in losses due to irreversible adsorption. Dialysis of lipid mixtures through rubber membranes in a non-polar

* Journal Paper No. J 4770 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1546.

solvent has also been used to separate polar and non-polar lipids[9,10]. The technique described here offers advantages of simplicity, speed, mild conditions and good resolution over the other methods mentioned.

Experimental

Lipid samples. Corn "lecithin", a preparation containing a variety of glycolipids and phospholipids from corn germ, was obtained from Corn Products Company, Argo, Ill. beef lung fat, from the VioBin Corporation, Monticello, Ill. Cholesterol stearate was synthesized and contributed by Mr. ALAN MA. Egg lecithin, cholesterol and triolein were standard commercial products.

Preparation of columns. Polystyrene beads with 2% divinylbenzene cross-linkages, 50–100 mesh, were obtained from the Dow Chemical Company, Midland, Mich. One hundred and thirty-nine g of this material was slurried in benzene and packed in a chromatographic column 4.0 cm in diameter to a height of 52 cm. The column was washed with about 1 l of benzene before the first use. Lipid samples were applied to the column in 5–10 ml benzene, and elution was carried out with the same solvent. Approximately 5 ml fractions were collected with a time-operated fraction collector.

Analysis of eluates. Weight curves were obtained by transferring 1 ml portions of each fraction into tared aluminum weighing dishes, evaporating the solvent under reduced pressure in a vacuum desiccator and reweighing. Phosphorus and galactose were estimated on 1 ml portions by the methods of HARRIS AND POPAT[11] and RADIN *et al.*[12] respectively. Infrared spectra of some samples before and after passage over the column were obtained as smears or Nujol mulls using a Perkin-Elmer Model 137 Infracord Spectrophotometer.

Results and discussion

An elution curve for corn "lecithin" is shown in Fig. 1. The mixture is resolved into two well-separated fractions, with all the phosphorus and carbohydrate in the first

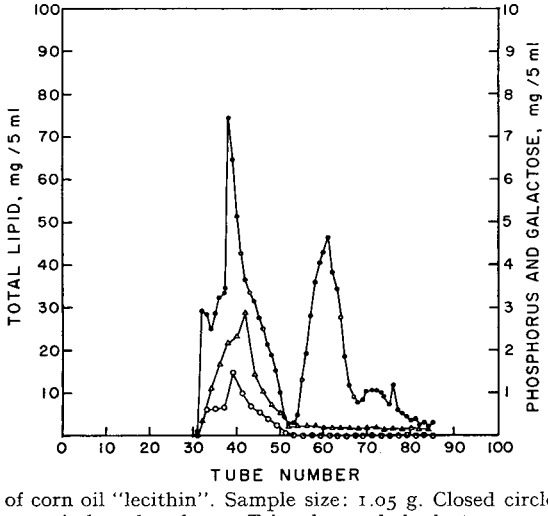


Fig. 1. Fractionation of corn oil "lecithin". Sample size: 1.05 g. Closed circles, total lipid. Open circles, phosphorus. Triangles, carbohydrate.

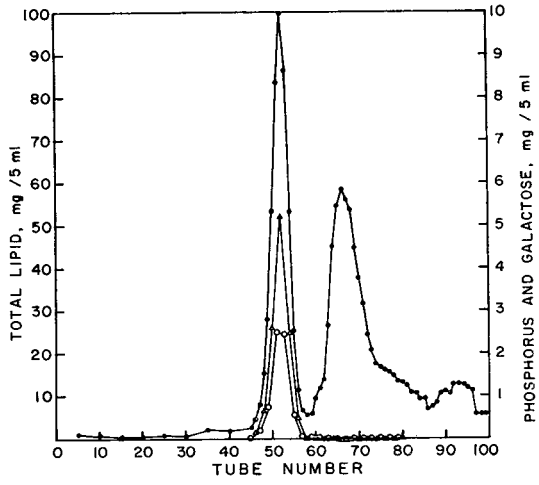


Fig. 2. Fractionation of beef lung fat. Sample size: 1.07 g. Symbols same as Fig. 1.

fraction, as expected from a gel filtration process. Fig. 2 shows an elution diagram for the fractionation of beef lung lipid on the same column. The complex lipids are again eluted first in a well-separated peak. The remaining lipids are partially resolved into three components. Qualitative Liebermann-Burchard tests on the contents of tubes 52, 68, 80 and 95 were positive only on the latter two samples, indicating partial resolution of sterols and sterol esters from triglycerides.

The fractionation of a model mixture containing egg lecithin, triolein, cholesterol stearate and cholesterol is illustrated in Fig. 3. I.R. spectra were obtained for materials from tubes 47, 61–63, 69 and 85 and compared with the spectra of the individual components of the model mixture. These fractions were identified in this way as egg

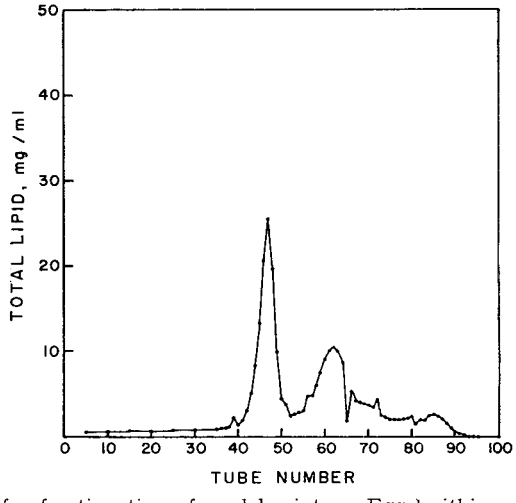


Fig. 3. Weight curve for fractionation of model mixture. Egg lecithin, 0.52 g; triolein, 0.45 g; cholesterol, 0.11 g; cholesterol stearate, 0.08 g.

lecithin, triolein, cholesterol stearate and cholesterol, respectively. Egg lecithin is seen to be well resolved from the remainder of the mixture, while triolein, cholesterol stearate and cholesterol are only partially separated from each other. The spectra of the samples before and after passage over the column were identical with two exceptions. The spectrum of egg lecithin before passage over the column had strong absorption at 1705 cm^{-1} due to free fatty acids. After passage over the column, this absorption was absent in the phosphatide fraction but was found instead in the spectrum of cholesterol stearate, indicating that free fatty acids and sterol esters are eluted together on this column.

The corn "lecithin", beef lung and egg lecithin samples all contained the brown pigment commonly found in lipid samples that have been exposed to the air. This brown material was eluted in each case with the complex lipids, the rest of the fractions being completely colorless. It was also observed that the flow rate of the column, which was set at 2–2.5 ml/min, decreased sharply while the complex lipids were emerging.

Recoveries of lipid samples were approximately 100%. The column is free-flowing with no appreciable tendency of the polystyrene beads to pack more tightly as the column is re-used repeatedly. A complete elution requires about four hours.

It is anticipated that this method will be useful for the preliminary separation of complex and simple lipids for preparative or analytical purposes. Further study of this procedure is now in progress.

Acknowledgement

This investigation was supported in part by research grant number GM-10457 from the United States Public Health Service.

Department of Biochemistry and Biophysics,
Iowa State University,
Ames, Iowa (U.S.A.)

C. L. TIPTON
J. W. PAULIS
M. D. PIERSON

[1] M. F. VAUGHN, *Nature*, 188 (1960) 55.
[2] M. FAURE AND J. LEGAULT-DEMARE, *Bull. Soc. Chim. Biol.*, 32 (1950) 509.
[3] J. LEGAULT-DEMARE AND M. FAURE, *Bull. Soc. Chim. Biol.*, 33 (1951) 1013.
[4] P. H. ELWORTHY, *J. Chem. Soc.*, (1959) 813.
[5] P. H. ELWORTHY AND D. S. MCINTOSH, *J. Pharm. Pharmacol.*, 13 (1961) 663.
[6] N. ROBINSON, *J. Pharm. Pharmacol.*, 13 (1961) 53.
[7] D. J. HANAHAN, *Lipide Chemistry*, John Wiley & Sons, New York, 1960, pp. 19–22.
[8] R. O. WEENICK, *New Zealand J. Sci.*, 2 (1959) 273.
[9] G. H. VAN BEERS, H. DE JONGH AND J. BOLDINGH, in H. M. SINCLAIR (Editor), *4th Intern. Conf. Biochem. Probl. of Lipids, Oxford, 1957, Proc.*, 1958, p. 43.
[10] D. EBENHAGEN AND H. BETZING, *J. Lipid Res.*, 3 (1962) 382.
[11] W. D. HARRIS AND P. POPAT, *J. Am. Oil. Chemists' Soc.*, 31 (1954) 124.
[12] N. S. RADIN, F. B. LAVIN AND J. R. BROWN, *J. Biol. Chem.*, 217 (1955) 789.

Received February 17th, 1964

Notes

Modified celite partition chromatography

In studies on the chromatography of metabolism fluids resulting from the action of *Acetobacter acetigenum* on glycerol, in a suitably defined medium, it became necessary to examine these fluids for their carbohydrate content in order to gain an insight into the mode of formation of bacterial cellulose. Application of Ultrasorb charcoal chromatography to these fluids by RAMAMURTI AND JACKSON[1] resulted in the isolation of eight water fractions, designated W_1 through W_8, and six aqueous ethanolic fractions A_1 through A_6. These fractions were further resolved by means of the celite partition chromatographic procedure of LEMIEUX, BISHOP AND PELLETIER[2] with modifications, the details of which are given below.

These experiments were carried out during the summer months when the variation between the day and night temperature was considerable, and it was observed that when celite columns were prepared by the slurry method of NEISH[3] and left running overnight (at the rate of 16 ml/h) a change in the equilibrium conditions of the solvent system (water-saturated *n*-butanol) seemed to occur. The appearance of the packed material at the top of the column underwent a progressive change rather as though it had become wetter. This effect was observed even under carefully controlled temperature conditions and after an overnight run extended some 2 cm down the column. Attempts to resolve one of the aqueous ethanolic fractions A_3 (approximate composition: glucose, fructose and traces of a pentose) on such a column were unsuccessful. When the chromatogram was stopped after 16 h and the column extruded and sprayed, no band was visible on the celite column. However, when the eluant (*ca.* 300 ml) was concentrated and examined by paper chromatography the picture was quite similar to that obtained from fraction A_3. The failure of the method to resolve fraction A_3, as seems probable, may be due to the leaching effect of water that might have separated from the solvent system (water-saturated *n*-butanol) during an overnight run. The following procedure was devised to counter the foregoing difficulty.

Experimental

One litre of re-destilled *n*-butanol was allowed to stand overnight in contact with 500 ml distilled water. A portion of the stationary phase was separated from the organic phase, and used in preparing the celite column, while the organic phase served as the developing medium in the procedure. 70 ml of stationary phase was absorbed on to dry acid washed celite No. 535 (70 g), which was then slurried with the developing phase; the celite column (32 × 2.8 cm) was constructed according to NEISH[3]. The sample to be fractionated, A_3, after deionisation with Zeo-Karb 225 and Deacidite E (10 % solution, wt./vol., in the stationary phase) was absorbed on dry celite No 535 (1 ml/g) and the resulting powder packed to the top of the packed celite column

which was just filled with the developing phase. The column was connected to a reservoir containing water-saturated *n*-butanol and the chromatogram was run for an hour to allow the developing solvent to percolate through the column. Then the column was disconnected from the reservoir and allowed to drain. When most of the solvent had drained off, the delivery end of the column was closed. Dry celite (5 g) was absorbed in 5 ml stationary phase and a thick slurry of this with the developing phase was packed tight on the top of the celite column, giving an extra height of 2.5 cm. The celite column was again connected to the reservoir containing water-saturated *n*-butanol and the chromatogram run for 16 h, the rate of flow of the solvent being 20 ml/h. Owing to the increased length of the column the change in the equilibrium conditions of the solvent system in the top 2 cm of the celite column did not impede the resolution of the sample to be fractionated. At the end of 16 h the celite column was disconnected from the reservoir and allowed to drain for 2 h. The column was extruded by a special technique that was developed during the course of this work.

Extrusion technique

Excess solvent was removed from the celite column by the application of gentle suction. The column was then placed in a slightly larger glass jacket tightly stoppered at one end by a rubber bung and compressed air was carefully blown through the celite column. As the celite was slowly dislodged, it was received into the wider glass jacket, while the tube which had contained the celite was gently withdrawn at the same time. The column of extruded celite material was carefully transferred to a large sheet of clean glass by means of a glass plunger, and then sprayed with alkaline potassium permanganate (1 % aqueous potassium permanganate containing 2 % sodium carbonate) through a mask in the form of a narrow slit. Eight bands of varying colour intensity appeared on the column. The portions of celite material, corresponding to the eight bands were sectioned with a clean knife and eluted with distilled water. Invariably the fourth band from the top of the celite column comprised nearly pure glucose. Enrichment of this component was sought by repeated partition of the deionised fraction A_3 on celite columns using the above procedure.

Acknowledgements

The authors wish to express their thanks to Prof. T. K. WALKER for initiating the inquiry and for interest in the work. One of us (K. RAMAMURTI) acknowledges the receipt of an Imperial Chemical Industries Research Fellowship.

College of Science and Technology, University of Manchester, Manchester (Great Britain)

K. RAMAMURTI*
C. P. JACKSON**

1 K. RAMAMURTI AND C. P. JACKSON, *J. Biol. Chem.*, 237 (1962) 2434.
2 R. U. LEMIEUX, C. T. BISHOP AND G. E. PELLETIER, *Can. J. Chem.*, 34 (1956) 1365.
3 A. C. NEISH., *Can. J. Res.*, B28 (1950) 535.

Received November 13th, 1963

* Present address: Professor of Chemistry, Faculty of Science, University of Libya, Tripoli, Libya.
** Present address: Bolton Technical College, Bolton, Lancs., Great Britain.

Partition chromatography of estrone, equilin and equilenin isolated from mares' urine*

Partition column chromatography has been extensively applied to the separation of both equine[1] and human[2–5] urines. HAENNI and associates[6], utilizing the sodium hydroxide impregnated Kieselguhr and benzene system of BANES and coworkers[1], separated estradiol-17β from estradiol-17α and the corresponding epimeric dihydroequilenins in a single chromatogram. HEARD and associates[7] applied the latter method to the separation of ketonic estrogens of mares' urine but required repetitive chromatography to separate estrone completely from its 7,8-unsaturated companion, equilin. This method was further modified by SAVARD AND BURDULIS[8] and provided satisfactory separation of estrone, equilin, equilenin and estradiol-17β in a single chromatogram, but left an intermixed portion of the estrone and equilin zones which amounted to approximately 13 to 18% of the two compounds.

The object of this report is to describe a further modification of the method of SAVARD AND BURDULIS which provides a good separation of estrone and equilin in a single chromatogram by means of a slow gradient increase in the elution power of the mobile phase.

Methods and materials

Solvents. All solvents were reagent grade, freshly distilled; the ligroin boiling range was 70–110°.

Celite. No. 535 of Johns-Manville and Co. was treated according to BAULD[5].

Estrogen mixtures. These were extracted from the urines of pregnant mares after solvolysis and fractionation of the phenolic extract into ketonic and non-ketonic fractions. The ketonic phenolic fraction was chromatographed on silica gel and the estrogens eluted in a semi-purified form with ethyl acetate in benzene in a step-wise manner.

Apparatus. The chromatography tube is 50 cm long by 1.8 cm uniform inside diameter, fitted at the lower end with a finely ground stopcock. The mixing chamber consists of a 5 l cylindrical bottle, stirred magnetically and connected to the chromatogram tube by means of an inverted U-shaped glass tube (2 mm I.D.); the connection between the chromatographic column and the connecting tube is sealed by a rubber stopcock and the solvent is thereby siphoned from the mixing chamber to the upper part of the chromatographic column. The reservoir consists of a 500 ml separatory funnel; with the stopcock open, the funnel is allowed to feed into a glass column whose rate of flow is regulated by a finely ground stopcock held in position by a stopcock tension clip. The solvent in this column is held at a constant level by the position of the stem of the separatory funnel; this constant level of solvent permits a steady rate of the flow of solvent into the mixing chamber. Both the lower tips of the chromatographic tube and the glass column are of the same inside diameter (1 mm) (Fig. 1).

Under normal operating conditions the reservoir and the glass column are filled with benzene, the mixing chamber is charged with 2000 ml 29% (v/v) benzene in ligroin.

* Supported in part by Grant No. C-4004, National Cancer Institute, United States Public Health Service.

By adjusting the flow of benzene in the mixing chamber at one-fourth the outflow of mixed solvent to the column, the desired solvent gradient, which is calculated from the equation described by LAKSHMANAN AND LIEBERMAN[9], is achieved.

Packing of the column. 29 g of celite are placed in a mortar and covered with 180 ml of 29 % (v/v) benzene in ligroin; exactly 30 ml of *N* sodium hydroxide solution

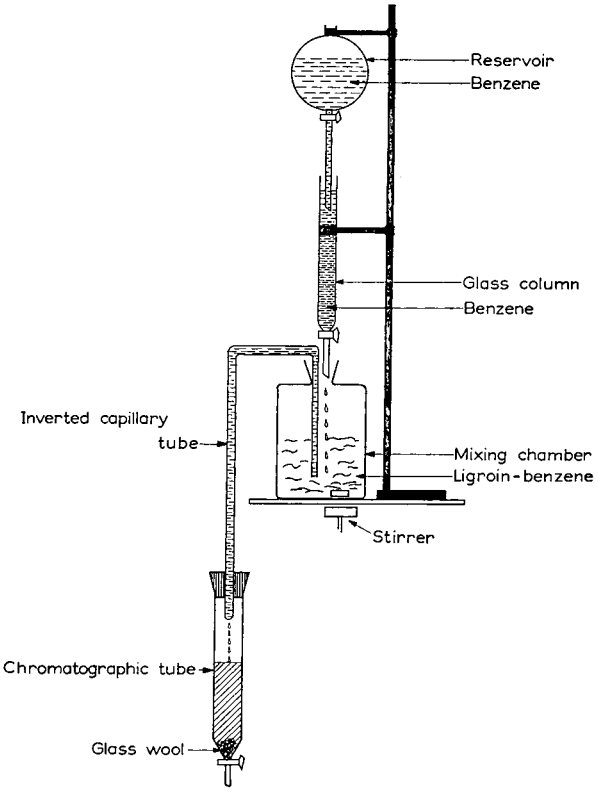


Fig. 1. Gradient elution chromatographic apparatus.

is distributed from a pipette over the Celite and the mixture is stirred for several minutes with a pestle until the celite appears uniformly wet. Meanwhile the chromatographic tube is one-quarter filled with solvent (29 % benzene in ligroin), a pad of glass-wool inserted and 1 g of celite, thoroughly impregnated with 0.5 water, is transferred to the column. The 29 g of sodium hydroxide impregnated celite is then transferred to the column and uniformly packed by means of a plunger as described by BAULD[10]. When complete the packed column is about 36 cm in height and the flow rate is 1.4 to 1.6 ml per minute.

Transfer of the steroid mixture to the column. The steroid mixture is dissolved in benzene and is mixed with 2 g of celite impregnated with 1 *N* sodium hydroxide solution (under ligroin–benzene). The solvents are allowed to evaporate and the steroid–celite mixture is transferred to the top of the prepared sodium hydroxide–celite column.

Running of the chromatogram. All chromatograms were run at 22–24° and 10 ml volumes of the eluate were collected. The steroids were quantitated in selected fractions by measuring their U.V. absorption in methanol.

Results and discussion

The initial composition of the mobile phase was selected after investigating the partition coefficients of estrone and equilin between 1 *N* NaOH solution and 50 and 30 % concentrations of benzene in various hydrocarbons (ligroin, heptane, methylcyclohexane). The level of 30 % benzene in saturated hydrocarbons affords approximately a two-fold difference in the partition coefficients of the two compounds in question. Accordingly the system 29–30 % benzene in ligroin was selected as the initial composition of the mobile phase.

It was observed that varying the volume of mobile phase in the mixing chamber and decreasing the concentration of benzene to 20 %, or both, did not improve the resolution of estrone and equilin zones, but actually increased considerably the threshold volume. Gradient variation in the concentration of benzene in the mobile phase, determined by the flow rates of solvent from reservoir to mixing chamber (R_1) and from mixing chamber to the column (R_2)[9] was found to give optimum resolution of estrone and equilin when the relationship of R_1/R_2 was $^1/_3$ or $^1/_4$ and R_2 was 1.4–1.6 ml/min. This provided on theoretical grounds a desirable concave shape to the solvent concentration curve, and in effect resulted in more symmetrically shaped elution curves for the principal estrogen zones than those obtained in the step-wise elutions described earlier[8].

Employing the conditions described in the methods section, up to 30 mg of a mixture of natural estrogens isolated from the urine of pregnant mares could be chromatographed on the sodium hydroxide impregnated celite partition column without any effect on the degree of separation or the symmetrical shape of the estrogens curves.

Endocrine Laboratory, University of Miami School of Medicine, Miami, Fla. (U.S.A.)

TAWFIK ELATTAR*
BURNICE BURDULIS
KENNETH SAVARD**

1 D. BANES, J. CAROL AND E. HAENNI, *J. Biol. Chem.*, 187 (1950) 557.
2 G. I. M. SWYER AND H. BRAUNSBERG, *J. Endocrinol.*, 7 (1951) ix.
3 J. BITMAN AND J. F. SYKES, *Science*, 117 (1953) 356.
4 E. H. AITKEN AND J. F. SYKES, *Science*, 117 (1953) 356.
5 W. S. BAULD, *Biochem. J.*, 63 (1956) 488.
6 E. O. HAENNI, J. CAROL AND D. BANES, *J. Am. Pharm. Assoc.*, 42 (1953) 167.
7 R. D. H. HEARD, R. JACOBS, V. O'DONNELL, F. G. PERON, J. C. SAFFRAN, S. S. SOLOMON, L. M. THOMPSON, H. WILLOUGHBY AND C. H. YATES, *Recent Prog. Hormone Res.*, 9 (1954) 383.
8 K. SAVARD AND B. BURDULIS, *Endocrinology*, 68 (1961) 411.
9 T. K. LAKSHMANAN AND S. LIEBERMAN, *Arch. Biochem. Biophys.*, 53 (1954) 258.
10 W. S. BAULD, *Biochem. J.*, 59 (1955) 294.

Received October 28th, 1963

* Present address: Physiologisch-chemisches Institut der Universität Bonn, Germany.
** Investigator, Howard Hughes Medical Institute.

Chromatography of streptothricins on carboxymethylcellulose

The streptothricins, a commonly occurring group of antibiotics, have been the subject of repeated attention on the part of investigators, owing to their high biological activity[1]. However, their isolation as individual compounds from their mixtures is as yet a rather complicated task. Although it has been shown in a number of studies that streptothricins can be fractionated by partition chromatography on cellulose[2–5], this method did not find extensive use as it is cumbersome and very inefficient.

In the course of studies of polymycin, a new antibiotic belonging to this group, we developed a convenient procedure for its separation based on ion exchange chromatography on carboxymethylcellulose (CMC). Later this was successfully applied to other streptothricin mixtures.

Fig. 1 gives the results of the analytical fractionation of five streptothricin antibiotics containing different numbers of components. To compare the data of several runs we recalculated the results as shown on the right-hand side of Fig. 1. This illustrates the close relation between the components of the various preparations.

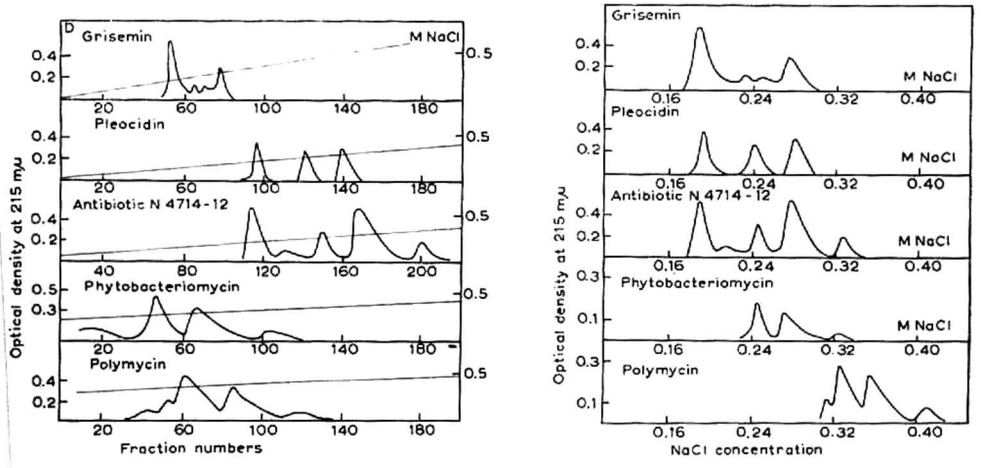


Fig. 1. Chromatography of five streptothricins on a CMC column. Column: 0.9 × 40 cm. Adsorbent: Na form of CMC, capacity 0.55 mequiv./g. Amount of substance: 20–35 mg hydrochloride in 0.5 ml H_2O. Rate: 15–20 ml/h. Fraction volume: 5.9 ml (grisemin); 4.8 ml (pleocidin); 5 ml (phytobacteriomycin); 4.6 ml (antibiotic No. 4714-12); 5.2 ml (polymycin). Eluent: NaCl solution with linear concentration gradient.

To show the reliability of the method a number of components were isolated from eluates with the aid of Amberlite IRC-50 in the Na form and were compared with the initial antibiotic preparations by paper chromatography (see Fig. 2). The right-hand side of Fig. 2 shows a comparison of the chromatographic behaviour of polymycin and antibiotic No. 4714-12, applied as a mixture, and of their constituents. It was found that the initial antibiotic preparations contain components with different R_F values. These values were found to cover mobilities of components of all chromatographically analysed streptothricins. Hence the polymycin + antibiotic No. 4714-12 mixture provides a convenient reference for the chromatographic analysis of new streptothricin preparations.

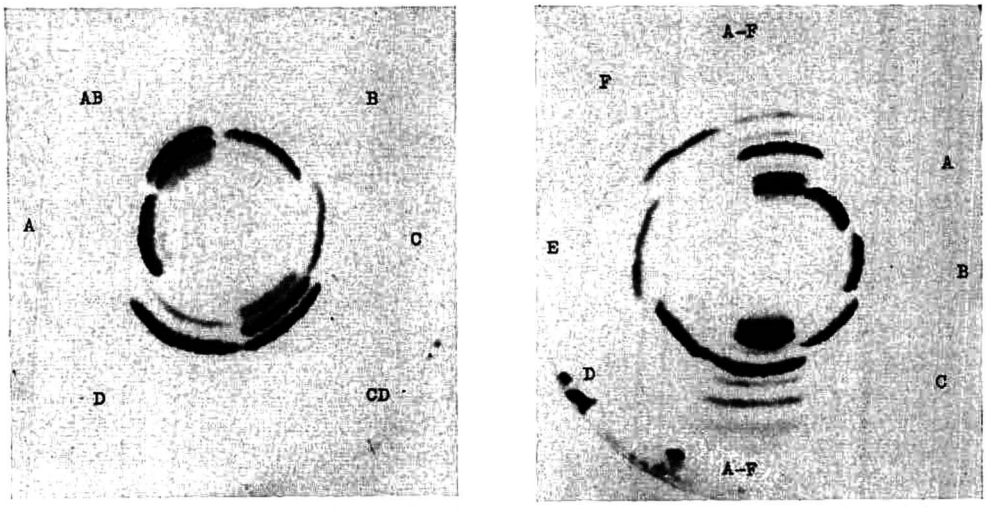


Fig. 2. Radial chromatograms of the isolated components and the initial streptothricins. Solvent: *n*-butanol–pyridine–acetic acid–water (15:10:3:12). Development: twice developed with the same solvent. Reagent: 0.25% ninhydrin in ethanol. Left-hand side: AB = initial polymycin; A, B = components of polymycin; CD = initial phytobacteriomycin; C, D = components of phytobacteriomycin. Right-hand side: A–F = mixture of polymycin and antibiotic No. 4714-12 applied as a single spot; A, B = components of polymycin; C, D, E, F = components of antibiotic No. 4714-12.

From the above results it can be seen that the proposed method has considerable advantages over partition chromatography on cellulose and, we believe, may find ready use for analytical and preparative purposes.

Institute for Chemistry of Natural Products, A. S. KHOKHLOV
U.S.S.R. Academy of Sciences, Moscow (U.S.S.R.) P. D. RESHETOV

[1] A. S. KHOKHLOV, M. M. SHEMYAKIN AND P. D. RESHETOV, *Streptothricins*, in M. M. SHEMYAKIN *et al.* (Editors), *Chemistry of Antibiotics*, Publ. House of USSR Acad. Sci., Moscow, 1961, p. 1200.
[2] M. I. HOROWITZ AND C. P. SCHAFFNER, *Anal. Chem.*, 30 (1958) 1616.
[3] U. F. ROUBEIN, *Dissertation Abstr.*, 22 (1961) 1390; *C.A.*, 56 (1962) 7429.
[4] A. W. JOHNSON AND J. W. WESTLEY, *J. Chem. Soc.*, (1962) 1642.
[5] H. TANIYAMA, F. MIYOSHI AND K. KAGEYAMA, *J. Pharm. Soc. Japan*, 82 (1962) 87; *C.A.*, 57 (1962) 963.

Received November 11th, 1963

Preparative separation of vitamins K_1 and K_3 from vitamins $K_{2(30)}$ and $K_{2(35)}$ by column chromatography

The earlier chromatographic methods for the isolation of vitamin K compounds entailed the use of Permutit[1], silica gel[2], Decalso[3], $MgSO_4$ and $ZnCO_3$[4]. The K vitamins are destroyed during chromatography on some adsorbents, such as Decalso or alumina[5] and as a consequence, prolonged chromatography must be avoided. Differ-

ent homologues of this vitamin can be identified by reversed-phase partition chromatography on silicone-coated paper[6]. MARTIUS[7] has isolated various forms of the K vitamins from extracts of animal tissue using the counter-current distribution technique.

Advances in silicic acid chromatography have facilitated the resolution of lipid mixtures and isolations of their components[8,9]. In the work to be presented, the authors have applied this method to the separation and isolation in a synthetic mixture of vitamin K_1(2-methyl-3-phytyl-1,4-naphthoquinone or phylloquinone), vitamin $K_{2(30)}$ (2-methyl-3-farnesylfarnesyl-1,4-naphthoquinone), vitamin $K_{2(35)}$ (2-methyl-3-farnesylgeranylgeranyl-1,4-naphthoquinone) and vitamin K_3 (2-methyl-1,4-naphthoquinone).

The solvent used in these experiments was either spectroanalyzed analytical grade *n*-hexane or redistilled petroleum ether (b.p. 60–70°). All other reagents were redistilled in glass with the exception of ethyl ether which was U.S.P. specification. Paper chromatography was carried out on silicone-coated Whatman No. 1 filter paper as described by GREEN AND DAM[6].

The preparation of the chromatographic column was similar to that described by HIRSH AND AHRENS[8]. The silicic acid was washed with 25 ml of ethyl ether, 50 ml of acetone–ethyl ether (1:1, v/v) and finally 40 ml of ethyl ether. Following the completion of these washes, the column was equilibrated for 16 h with *n*-hexane. During the pretreatment and fractionation procedures, the jacketed chromatographic apparatus was maintained at a constant temperature of 25°. The amounts used in the chromatographic runs ranged from 30 to 97 mg. Preliminary adsorption isotherm studies indicated that the K vitamins could be obtained in micro quantities.

The first chromatographic elution scheme (called scheme 1) was started with 1% ethyl ether in *n*-hexane followed by two subsequent stepwise developing solutions. These were a 4% solution of ethyl ether in *n*-hexane and 100% ethyl ether which was employed to clear the column. However, in order to obtain better resolution of the four-component mixture, a second scheme (called scheme 2) was employed by starting with a concentration of 0.5% ethyl ether in *n*-hexane followed by 1% and 4% ethyl ether in *n*-hexane respectively. Again, 100% ethyl ether was used to clear the column.

The eluted fractions were collected in 25 ml volumes at a flow rate of approximately 0.5 ml/min. Immediately after elution from the column, ultra-violet spectroanalysis was performed on a Beckman DK-2 recording spectrophotometer. Subsequently, the solvent was evaporated from the tubes at 50° using air jets and the weight of each fraction determined gravimetrically. The residues were examined by infra red spectrophotometry on a Perkin Elmer infra-red apparatus model 21 for further characterization.

When vitamins K_1, $K_{2(30)}$, $K_{2(35)}$ and K_3 were chromatographed together, employing scheme 1, 98% of the total mixture was recovered but the first peak was heterogeneous and was produced by vitamins K_1, $K_{2(30)}$, $K_{2(35)}$, as determined by reversed-phase partition chromatography on silicone-coated paper. Ultra-violet spectroanalysis and partition chromatography revealed that the second peak was caused by vitamin K_3.

An increased resolution of the four-component mixture was accomplished employing scheme 2 and this is shown in Fig. 1. Reversed-phase partition chromatog-

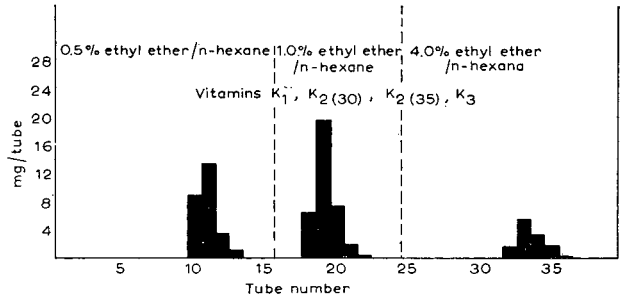


Fig. 1. Chromatography of vitamins K_1, $K_{2(30)}$, $K_{2(35)}$ and K_3 employing elution scheme 2: mg/tube *versus* tube number. First peak: vitamin K_1; second peak: vitamins $K_{2(30)}$ and $K_{2(35)}$; third peak: vitamin K_3.

raphy indicated that the first peak, eluted with 0.5 % ethyl ether in *n*-hexane, was homogeneous and was phylloquinone. The second peak, eluted with a 1.0 % solution was a mixture of vitamin $K_{2(30)}$ and $K_{2(35)}$, with the former more concentrated in the front of the peak and the latter more concentrated in the tail of the peak. The third peak consisted solely of menadione. Infra-red analyses supported these conclusions. So far, the employment of gradient elution has failed to improve the resolution of this four-component system.

It appears, therefore, that the procedure described is useful for the separation of vitamins K_1 and K_3 as homogeneous fractions from the two vitamins, $K_{2(30)}$ and $K_{2(35)}$, and shows promise for the isolations of these substances from a lipid mixture isolated from tissues and body fluids. It must be kept in mind, however, that these K homologues may behave differently in the presence of other lipids. Such studies are currently being undertaken both with synthetic lipid mixtures and lipid extracts of biological material.

The authors wish to express their gratitude to Dr. OTTO ISLER for his generous gift of vitamin $K_{2(30)}$ and vitamin $K_{2(35)}$. This investigation was supported by Medical Research Council of Canada Grant Number CG-24.

Department of Physiology and Pharmacology, R. LOSITO
University of Saskatchewan, Saskatoon (Canada) G. J. MILLAR

1 R. J. WOODS AND J. D. TAYLOR, *Can. J. Chem.*, 35 (1957) 941.
2 H. NOLL AND H. BLOCH, *J. Biol. Chem.*, 214 (1955) 251.
3 S. B. BRINKLEY, D. W. MACCORQUODALE, S. A. THAYER AND E. A. DOISY, *J. Biol. Chem.*, 130 (1939) 219.
4 P. KARRER, A. GEIGER, R. LEGLER, A. RÜEGGER AND H. SALMON, *Helv. Chim. Acta*, 22 (1939) 1464.
5 J. P. GREEN AND H. DAM, *Acta Chem. Scand.*, 8 (1954) 1093.
6 J. P. GREEN AND H. DAM, *Acta Chem. Scand.*, 8 (1954) 1341.
7 C. MARTIUS, *Am. J. Clin. Nutr.*, 9 (1961) 97.
8 J. HIRSH AND E. H. AHRENS, *J. Biol. Chem.*, 233 (1958) 311.
9 E. W. LIS, J. TINOCO AND R. OKEY, *Anal. Biochem.*, 2 (1961) 100.

Received October 28th, 1963

Changes occurring in active alumina on storage

We have examined the chromatographic behaviour of rotenone on neutral alumina in ether–petroleum ether solvent mixture. Under these conditions part of the rotenone is irreversibly adsorbed by the alumina and a study of this phenomenon forms the basis of this communication.

Experimental

Materials. Active alumina was prepared from commercial material (Peter Spence, Type H, γ-Al_2O_3), by acidification (pH 2) of an aqueous suspension followed by washing until the aqueous supernatant was neutral. The alumina was then filtered off and dried on plates in an oven at 100° for 3 days. The "dry" active alumina contained approximately 5 % water and prior to use water (7 % v/w) was added and the sample of deactivated alumina rolled in a closed container for 2 h. The solvents used were dried by distillation, except the solvent ether which was distilled from sodium metal. The rotenone used had m.p. 161.5°, λ_{max} 237 mμ, ε_{max} 14,580.

Chromatographic method. A column (15 cm × 1.5 cm) was packed with alumina (50 g Grade III), using solvent ether–petroleum ether (b.p. 40–60°) (1:1) mixture (100 ml) in the usual manner. A solution of rotenone (60 mg) in the eluent mixture (25 ml) was then introduced onto the column followed by elution with the same eluent mixture. Fractions (10 ml) of the eluate were collected and an aliquot (0.05–0.5 ml) of each fraction was evaporated with a stream of nitrogen and then dissolved in spectroscopic ethanol (95 %, 10 ml) by warming in a water bath at 40°. The optical density at 237 mμ was determined for each diluted fraction. The amount of rotenone recovered from the column was calculated from the expression:

$$\text{rotenone (mg)} = \Sigma[(x - y) \times \text{dilution factor}] \times \frac{3.94 \times 1000}{14{,}580}$$

where x = optical density of diluted aliquots,
y = optical density of diluted aliquots of eluent mixture.

In later experiments, it was found more convenient to collect a bulk fraction (250 ml) after 100 ml of eluate had left the column. This fraction contained all the rotenone.

Storage of alumina. The dried active alumina was removed from the oven (time = zero), and stored in a well-filled closed glass container. At intervals, 50 g was removed, deactivated with water and used for the chromatography of rotenone. Fig. 1 shows the variation in the rotenone recovery obtained using deactivated alumina prepared from two batches of "dry" alumina stored for various time intervals. The general shape of the curves obtained for the two batches was similar although the positions of the maxima and minima differed considerably.

Discussion

We have found that when rotenone is chromatographed on alumina in dry ether–petroleum ether solvent mixture (1:1), part of the rotenone is irreversibly adsorbed (decomposed). The decomposition product remained on the column since the ultraviolet absorption characteristics of the eluate were those of rotenone, and further-

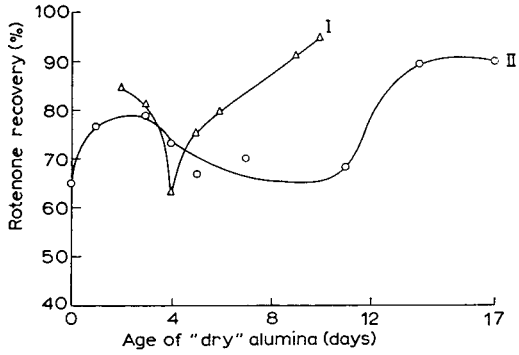


Fig. 1. Rotenone recoveries from deactivated stored "dry" alumina.

more the elution curve was symmetrical and did not show tailing (see Fig. 2). The recoveries of rotenone from the column were quantitative when solvent ether previously saturated with water was used in the solvent mixture.

The activity of alumina is directly related to its water content[1] since the addition of water to dry active alumina decreases the activity of the alumina by reducing the number of available adsorption sites[2]. The solvent used in our work has a high affinity for water and presumably some of the physically adsorbed water molecules on the alumina surface are removed, exposing an active surface on which part of the rotenone is irreversibly adsorbed. This view receives support from the work of SNYDER who has shown that non-chemically bound water in samples of alumina containing more than 4–5 % water may be partially removed by a weaker hydrophilic eluent such as benzene[2].

It has been further noted that the extent to which the rotenone is irreversibly adsorbed by the alumina is dependent upon the age of the "dry" alumina from the

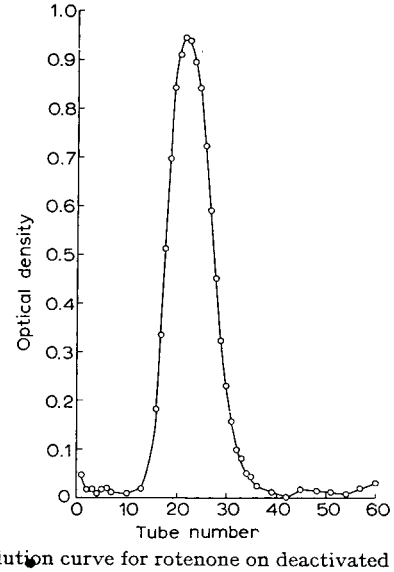


Fig. 2. Elution curve for rotenone on deactivated alumina.

time of its preparation (see Fig. 1). KIPLING AND PEAKALL have shown that active alumina (γ-Al_2O_3) in contact with water vapour irreversibly adsorbs water molecules to give a gibbsite-like surface ($Al_2O_3 \cdot 3H_2O$)[3]. We suggest that the "dry" alumina which contains a small proportion of water (5 %) undergoes similar changes at the surface on storage, and that as hydration proceeds the dipolar character of the (–Al–O–) bonds at the surface are altered, and the dipolar attractive forces between the surface of "dry" alumina and the added water molecules are modified accordingly. Elution of the water molecules at the surface by hydrophilic solvent mixture will become easier as these attractive forces decrease, leading to increased rotenone decomposition and *vice versa*. However, this explanation does not account for the complex nature of the curves in terms of the species γ-Al_2O_3, AlOH(:O) and $Al_2O_3 \cdot 3H_2O$, and the phenomenon is not clearly understood.

Now this interpretation of our results requires further comment since from an examination of free moisture in stored deactivated alumina WOHLLEBEN[4] has concluded that the chromatographic activity of alumina is not influenced by storage. We have reconciled these two views by showing that although the irreversible adsorption of rotenone is very sensitive to the changes occurring at the alumina surface on storage, the activity of the alumina to a reversible adsorption process is unchanged by storage.

Stored "dry" alumina was deactivated with water in the usual manner and then washed with the same volume of dry ether–petroleum ether (1:1) mixture (100 ml) as was used to prepare the column in the experiments with rotenone. The alumina was then air-dried and the activity determined by measuring the distance travelled by the front of the Sudan-Yellow dye band in the BROCKMANN method of grading[5]. A control was conducted when the activity of the deactivated alumina was directly determined by the BROCKMANN method (see Table I). The preliminary washing of the

TABLE I

BROCKMANN GRADING OF STORED "DRY" ALUMINA AFTER DEACTIVATION

Age of "dry" alumina (days)	*1*	*1½*	*3*	*5*	*7*	*10*
Distance travelled by dye-front (cm)						
Unwashed	3.5	3.2	3.3	3.9	3.6	3.3
Washed	3.2	3.4	3.0	3.3	3.8	3.3

alumina with the ether–petroleum ether solvent did not appreciably increase its activity towards reversible adsorption of Sudan-Yellow, and furthermore the activity was independent of the age of the "dry" alumina.

The rotenone was readily eluted from the column and not subjected to the more fully exposed alumina surface to be expected after the passage of all the solvent used in the experiment (500 ml). It seemed of interest to amplify the previous experiment to see how a more fully exposed alumina surface affected the reversible and irreversible adsorption process being studied.

Rotenone was passed down a freshly-used column from which a 89 % recovery of rotenone had been noted in the usual manner. All of the rotenone was irreversibly adsorbed on the column. Control experiments without the rotenone showed that the

BROCKMANN grading of the alumina could have differed only slightly before and after the first batch of rotenone was passed down the column since the distances travelled by the Sudan-Yellow dye were 3.7 and 3.3 cm respectively.

The amount of alumina surface exposed by the solvent in the first run was sufficient to considerably affect the irreversible adsorption of rotenone but insufficient to appreciably affect the average activity of the alumina as determined by the BROCKMANN method.

It can be concluded from this work that whereas reversible adsorption processes are unaffected by the changes occurring in "dry" alumina on storage, the chromatographic recoveries of small quantities of materials which are labile to alumina may be profoundly affected when dry strongly hydrophilic solvents are being used.

Welsh School of Pharmacy, Welsh College of Advanced Technology, Cardiff, S. Wales (Great Britain)

E. BRIERLEY
H. J. SMITH

[1] E. STAHL, *Chemiker-Ztg.*, 85 (ii) (1961) 371.
[2] L. R. SNYDER, *J. Chromatog.*, 6 (1961) 22.
[3] J. J. KIPLING AND D. B. PEAKALL, *J. Chem. Soc.*, (1957) 834.
[4] G. WOHLLEBEN, *J. Chromatog.*, 1 (1958) 271.
[5] H. BROCKMANN AND H. SCHODDER, *Ber.*, 74 (1941) 73.

Received November 26th, 1963

A simple elution device for the automatic stepwise chromatography of lipids*

Several reproducible column chromatographic techniques are available which employ discrete changes in mobile phase composition for the analysis of lipid extracts. However, the frequent attention of the operator is required for the addition of each discrete mobile phase. Also, inconsistency in the addition of the phase solvents may decrease the reproducibility of these methods.

A simple reservoir apparatus was designed for use in conjunction with an automatic fraction collector to facilitate the use of these methods. This device is best employed with a chromatographic system requiring five or less mobile phase changes.

The different solvent phases are placed into each numbered reservoir, as illustrated in Fig. 1. The reservoirs are filled in reverse order, starting with number 5. If all five reservoirs are not needed for the system utilized, the remaining unused upper reservoirs can be left empty without affecting the efficiency of the device. After the required reservoirs are filled, the center capillary tube is cleared of mixed solvents by opening the stopcock carefully to allow flushing of the tube with solvent from the uppermost reservoir. The device is then connected to the column so that the standard taper outlet enters onto the column. When the stopcock is opened, the mobile phase

* This investigation was supported in part by Public Health Service Research Grant 4432 from the National Institute of Arthritis and Metabolic Diseases, Contribution No. 38, Kansas Agricultural Experiment Station.

in reservoir 1 is the first to enter the column, followed in order by those in reservoirs 2, 3, 4 and 5. Although the apparatus is designed on a gravity flow principle, a simple manifold fitted to the tops of the capillary tubes enables the system to be run under pressure. After setting up the reservoir device with the column and automatic fraction collector, no further attention is required by the operator.

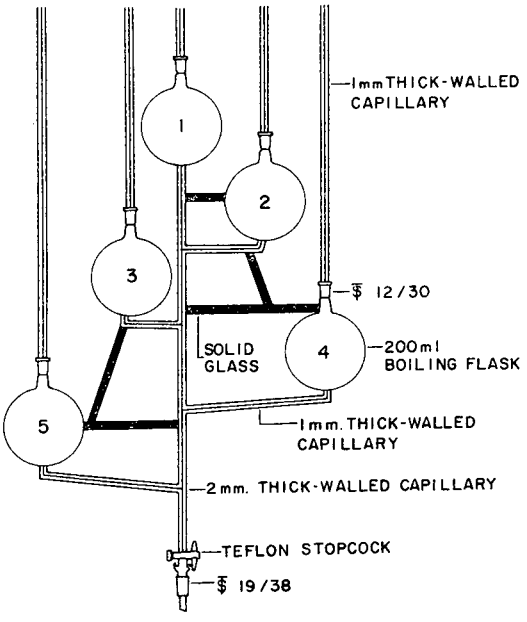


Fig. 1. The elution device. The order of phase delivery is from reservoirs 1, 2, 3, 4 and 5, respectively

Colored indicators were used initially to check the uniformity of flow from the reservoir. Only an insignificant amount of phase mixing was noted, and this was a constant, reproducible factor for all runs. This apparatus has been used in our laboratory with excellent reproducibility in conjunction with the FILLERUP AND MEAD procedure[1], which utilizes five mixed solvent eluting fractions and a silicic acid column for the separation of lipid mixtures.

*Department of Biochemistry, Kansas State University, Manhattan, Kans. and Department of Chemistry, University of Massachusetts, Amherst, Mass. (U.S.A.)**

D. R. BRUSCA
A. M. GAWIENOWSKI

[1] D. L. FILLERUP AND F. MEAD, *Proc. Soc. Exptl. Biol. Med.*, 83 (1953) 574.

Received December 20th, 1963

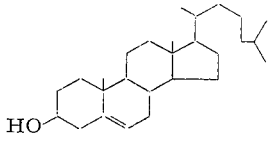
* Present address of the authors.

The separation of sterols and corresponding stanols by thin-layer chromatography

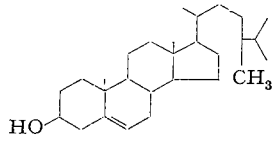
The sitosterols, such as β-sitosterol, occur very often in plants as complex mixtures with the corresponding stanols, mixtures which are difficult to resolve. Studies carried out on the constituents of Israeli peat revealed the presence of an inseparable mixture of β-sitosterol and β-sitostanol. Similar observations have been made by IVES AND O'NEILL[1] with Canadian peat moss (Sphagnum) and by MCLEAN, RETTIE AND SPRING[2] with Scottish peat. In order to prove the presence of sitostanol, the mixture is usually oxidized with chromium trioxide in acetic acid, and the β-sitostanone thus formed is identified. The disadvantages of this method are that the rupture of the double bond in sitosterol present produces polar oxidation products, and that the procedure is time consuming. As the mobilities of sterols and the corresponding stanols are so similar that chromatography makes them practically inseparable (Table II), the applicability of a method first suggested by CARGILL[3] for the separation of cholesterol from related stanols and stanones was investigated.

By this method the sterol mixture is brominated, the stanol remaining unchanged so that it can be separated chromatographically from the brominated sterol. Whilst FABRO[4] used reversed phase paper chromatography for this separation we followed CARGILL[3] in applying thin-layer chromatography. Separations were carried out on plates covered with silica gel G or alumina G (Merck), in 4-solvent systems (see below). The results are summarized in Table I. This method can also be used for the detection of traces of unreduced sterols in products which had been subjected to catalytic reduction.

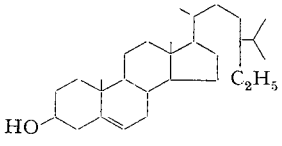
For compasorin, the free sterols were run on alumina G plates, solvent system No. 4 serving as the developer. The R_F values are summarized in Table II.



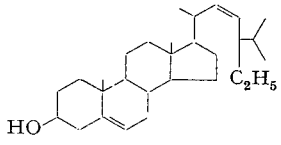
cholesterol



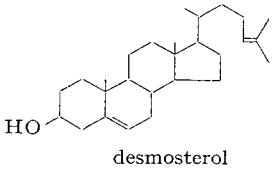
campesterol



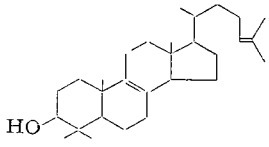
β-sitosterol



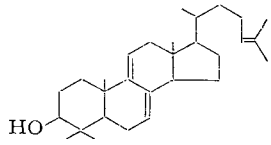
stigmasterol



desmosterol



lanosterol



agnosterol

TABLE I

R_F VALUES OF STANOLS AND BROMINATED STEROLS

The following mixtures were separated: campesterol–campestanol, cholesterol–cholestanol, cholesterol–coprostanol, cholestanol–coprostanol, cholesterol–cholestanol–coprostanol, lanosterol–dihydrolanosterol, stigmasterol–stigmastanol, β-sitosterol–β-sitostanol.

No.	Sterol (after bromination)	R_F in solvent system					
		1	2		3	4	
		Silica Gel G	Silica Gel G	Alumina G	Silica Gel G	Silica Gel G	Alumina G
1	Campesterol						0.69
2	Campestanol						0.51
3	Cholesterol	0.81	0.78	0.63	0.31	0.44	0.68
4	*allo*-Cholesterol	0.95	0.92	0.77	0.43	0.58	0.82
5	Desmosterol	0.82	0.79			0.47	0.69
6	Cholestanol	0.62	0.60	0.44	0.19	0.27	0.51
7	Coprostanol	0.76	0.66				0.69
8	β-Sitosterol	0.79	0.80	0.63	0.32	0.43	0.68
9	Stigmasterol	0.80	0.76	0.62		0.44	0.68
10	β-Sitostanol (= Stigmastanol)	0.60	0.59	0.44	0.18	0.27	0.50
11	Lanosterol	0.57	0.77			0.59	0.81
12	Dihydrolanosterol	0.50	0.55				0.65
13	Agnosterol	1.00	1.00				0.94

Experimental

Preparation of plates. The suspension required for five plates (20 × 20 cm) was prepared by shaking 50 g of alumina G and 100 ml of water or 30 g of silica gel G and 60 ml of water for 30 sec; it was then spread on the plates with a Desaga thin-layer applicator to give a layer of 0.25 mm thickness. The plates were allowed to dry for 60 min at room temperature and for 30 min at 125°. After cooling, they were placed in a vacuum desiccator.

TABLE II

R_F VALUES OF FREE STEROLS ON ALUMINA G, SOLVENT SYSTEM NO. 4

No.	Sterol	R_F	Colour with	
			$SbCl_3$	$SbCl_5$
1	Campesterol	0.50	Pink	Brown
2	Campestanol	0.51	—	Brown
3	Cholesterol	0.50	Pink	Brown
4	Cholestanol	0.51	—	Brown
5	Coprostanol	0.69	—	Brown
6	*allo*-Cholesterol	0.53	Pink	Brown
7	21-*nor*-Cholesterol[5]	0.52	Pale violet	Brown
8	Desmosterol	0.50	Brown	Brown
9	Lanosterol	0.78	Yellow	Brown
10	Dihydrolanosterol	0.77	Yellow	Brown
11	Ergosterol	0.52	Violet	Brown
12	Agnosterol	0.95	Yellow	Brown
13	β-Sitosterol	0.50	Pink	Brown
14	β-Sitostanol	0.49	—	Brown
15	Stigmasterol	0.52	Pink	Brown

Development. The samples were dissolved in chloroform (1 mg/1 ml of chloroform) and applied with micropipettes along a line 2 cm above the rim of the plate. Development was accomplished in a saturated chamber in four solvent systems (ratios in v/v), (1) benzene–ethyl acetate (2:1), (2) benzene–ethyl acetate (4:1), (3) benzene–ethanol (19:0.2), and (4) benzene–ethanol (19:0.4).

The experiments were performed at room temperature (23–25°). Usually 60 min were required for the solvent to reach a distance of about 12 cm. The plates were removed and the solvent was allowed to evaporate.

Detection. When the plates are heated at 120° for 15 min, the brominated products appear as green spots. Spraying with a saturated chloroform solution of antimony trichloride gave blue spots which turned green and finally grey, except for lanosterol and dihydrolanosterol which gave a yellow colour. The sterols were revealed as brown spots only after spraying with a solution of antimony pentachloride (30 % in chloroform).

Acknowledgement

The authors wish to thank Dr. J. AVIGAN, Dept. of Biochemistry, School of Medicine, the Hebrew Univeristy, for the samples of desmosterol, ergosterol, lanosterol and dihydrolanosterol.

Department of Organic Chemistry,
Hebrew University, Jerusalem (Israel)

R. IKAN
S. HAREL
J. KASHMAN
E. D. BERGMANN

[1] D. A. J. IVES AND A. N. O'NEILL, *Can. J. Chem.*, 36 (1958) 436.
[2] J. MCLEAN, G. H. RETTIE AND F. S. SPRING, *Chem. Ind. (London)*, (1958) 1515.
[3] D. I. CARGILL, *Analyst*, 87 (1962) 865.
[4] S. FABRO, *J. Lipid Res.*, 3 (1962) 481.
[5] SH. BLUM, *Ph. D. Thesis*, Jerusalem, 1962.

Received October 2nd, 1963

J. Chromatog., 14 (1964) 504–506

Separation of corticosteroids by thin-layer chromatography on silica gel plates containing tetrazolium blue

Tetrazolium salts, *e.g.* triphenyltetrazolium chloride, tetrazolium blue, etc., are useful reagents for the detection of corticosteroids. Tetrazolium salts in alkaline media are transformed to coloured formazans by corticosteroids, and this is the reaction that serves as the basis of their detection.

When thin-layer chromatography had become generally known and practised, several authors, *e.g.* METZ[1] and NISHIKAZE AND STAUDINGER[2], made use of this reaction after separation by thin-layer chromatography. Unfortunately, the sensitivity of the tetrazolium reaction shown in paper chromatography, *i.e.* 0.2 to 0.5 μg in the case of tetrazolium blue, could not be attained by spraying the surface of the

thin-layer chromatographic plates with an alkaline tetrazolium solution. This sensitivity, however, is attainable when tetrazolium blue is added to the silica gel before the preparation of the thin-layer plates. The details of the method used in these laboratories can be given briefly as follows:

Materials

(a) Tetrazolium blue (REANÀL, Budapest), (b) Kieselgel HF_{254} (MERCK, Darmstadt), (c) Solution of sodium alcoholate (10 g reagent grade NaOH dissolved in 100 ml 60 % methanol), (d) Solution of formic acid (2.0 ml of conc. formic acid added to 100 ml of methanol), (e) Solution of "Neatan" (MERCK, Darmstadt).

Method

Tetrazolium blue (100 to 200 mg) and 30 g of Kieselgel HF_{254} are thoroughly mixed, then about 100 ml distilled water is added and the mass is homogenized. A layer of about 0.2 mm thickness is spread with a DESAGA-type equipment on six glass plates of 20 × 20 cm, which are then left to dry at room temperature for at least 24 h.

The corticosteroids are dissolved in ethanol, or chloroform, or dichloromethane, and spotted on the thin layer. The solvent systems chloroform—ethanol (90:10), or dichloromethane–benzene—acetone—ethanol (75:10:10:5), are used to develop the chromatogram (40 to 80 min). These solvent mixtures do not extract tetrazolium blue from the support.

After development of the chromatogram the surface of the plate is sprayed with sodium alcoholate solution. When the coloured spots of the formazan compounds have become visible, further transformation of tetrazolium blue to formazan within the alkaline medium is arrested by careful spraying with a methanolic solution of formic acid. The plates are then allowed to dry, at room temperature, for about 20 to 30 minutes, and sprayed with Neatan solution to fix them. Transfer can be made by the usual method. In the acid solution and without fixation, the silica gel flakes off the plates when completely dry, *i.e.* within one or two hours.

Discussion

In addition to giving greater sensitivity, this method is advantageous because it also allows a more uniform distribution of the tetrazolium reagent. The increased sensitivity might be explained by a more intensive contact being possible between the tetrazolium reagent and the steroid molecules situated at greater depths, as well as those present at the surface.

It is suggested here that, in other cases also, addition of the reagent to the silica gel substrate will bring about an increase in the sensitivity of thin-layer chromatographic methods.

Research Department, National Institute of Rheumatic Diseases and of Balneology, Budapest (Hungary)

PÁL VECSEI (WEISZ)
VERONIKA KEMÉNY
ÁGOTA GÖRGÉNYI

[1] H. METZ, *Naturwiss.*, 48 (1961) 569.
[2] O. NISHIKAZE AND HJ. STAUDINGER, *Klin. Wochschr.*, 40 (1962) 1014.

Received October 9th, 1963

Separation and radioassay of fecal cholesterol and coprosterol using thin-layer chromatography*

Separation of fecal neutral sterols by column chromatography[1–4], and by gas–liquid chromatography[5,6] has been reported by several groups of investigators. Separation of synthetic compounds by thin-layer chromatography (TLC) was also described[7,8]. In the present paper, the quantitative separation of fecal cholesterol and coprosterol by TLC is reported.

TLC was carried out according to the procedure of STAHL[9] on 20 × 20 cm plates, 275 mμ thick. The plates were developed with a solvent system containing toluene and ethyl acetate (9:1, v/v) until the solvent front had migrated 12 cm from the origin. The development time was approximately 40 min. The plates were sprayed with a solution of phosphomolybdic acid (10% w/v) and heated to 110° for detection of sterols. Each unsprayed gel area, corresponding to a migration zone, was introduced into a liquid scintillation counter vial, and ^{14}C and ^{3}H activities were determined.

Coprosterol-^{14}C was prepared in the following manner: cholesterol-4-^{14}C (50 mC/mmol) was suspended in ethyl ether and added to 50 ml of isotonic NaCl solution. The ether was evaporated and the mixture was autoclaved at 125° for 15 min. An aliquot of normal human feces was transferred to the flask and was incubated at 37° with slow shaking for 6 days under nitrogen. The incubate was lyophilized and the powder was extracted in a Soxhlet extractor with ethyl alcohol. Saponification was carried out by KOH, and neutral sterols were extracted with petroleum ether.

TABLE I

AVERAGE C.P.M. PER ZONE (AND STANDARD ERROR OF MEANS) OF TLC SEPARATED NEUTRAL STEROL EXTRACT OF HUMAN FECES AFTER THE ADDITION OF CHOLESTEROL-7α-^{3}H AND COPROSTEROL-4-^{14}C RECOVERED FROM ZONES CORRESPONDING TO CHOLESTEROL AND COPROSTEROL

Amount μl	*Number of spots*		*Cholesterol zone*		*Coprosterol zone*	
			^{3}H *(c.p.m.)*	^{14}C *(c.p.m.)*	^{3}H *(c.p.m.)*	^{14}C *(c.p.m.)*
5	4	Average	1263 ± 36	0	10 ± 1.5	203 ± 8
		% Distribution	97	—	0.8	100
10	4	Average	2744 ± 55	0	23 ± 3.5	463 ± 9
		% Distribution	98	—	0.8	99.6
20	13	Average	5155 ± 86	0	28 ± 3.6	872 ± 16
		% Distribution	98.7	—	0.5	99.6

The petroleum ether was washed with water, concentrated and chromatographed twice in the system described. The zone corresponding to coprosterol was cut out, the silica gel recovered and extracted three times with ethanol and acetone (1:1, v/v) This procedure resulted in a 30–40% conversion of cholesterol-zone radioactivity to

* Supported by Grant H-7188 from the National Institutes of Health, U.S. Public Health Service.

coprosterol-zone radioactivity in the present system. When the latter material was subjected to gas–liquid chromatography (by Drs. E. MOSBACH and S. SHEFER) a single homogeneous peak was obtained in the zone corresponding to coprosterol. Cholesterol-7α-^{3}H (225 mC/mmol) and coprosterol-^{14}C were added to the "cold" cholesterol–coprosterol mixture and to neutral sterol extract of normal human feces before application to the chromatoplates.

The R_F values of the various sterols investigated were: cholesterol 0.30; coprosterol 0.40; epi-coprosterol 0.42; coprostenone 0.41; Δ^7-cholesten-3β-ol 0.26; cholestanol 0.27; 7-dehydrocholesterol 0.28; β-sitosterol 0.30; stigmasterol 0.31; 4-cholesten-3-one 0.50.

All cholestane compounds migrated together with cholesterol, except 4-cholesten-3-one, and all coprostane compounds were recovered with coprosterol. Table I shows the radioactivity recovered from the chromatographed stool extract. The average overlap of the two labels carried by the two compounds varied between 0 and 0.8 %. Since the larger majority of fecal neutral sterols consists of coprosterol and cholesterol, the method described seems satisfactory for the rapid separation of these two classes of material.

Department of Medicine, The Long Island Jewish Hospital, New Hyde Park, N.Y. (U.S.A.) and Department of Pathology, New York University School of Medicine, New York, N.Y. (U.S.A.)

PAUL SAMUEL
MORTON URIVETZKY
GABOR KALEY

1 W. W. WELLS, D. L. COLEMAN AND C. A. BAUMANN, *Arch. Biochem. Biophys.*, 57 (1955) 437.
2 R. S. ROSENFELD, D. K. FUKUSHIMA, L. HELLMAN AND T. F. GALLAGHER, *J. Biol. Chem.*, 211 (1954) 301.
3 H. DANIELSSON, *Biochim. Biophys. Acta*, 27 (1958) 401.
4 J. HIRSCH AND E. H. AHRENS, *J. Biol. Chem.*, 233 (1958) 311.
5 J. D. WILSON, *J. Lipid. Res.*, 2 (1961) 350.
6 W. W. WELLS AND M. MAKITA, *Anal. Biochem.*, 4 (1962) 204.
7 J. AVIGAN, D. S. GOODMAN AND D. STEINBERG, *J. Lipid Res.*, 4 (1963) 100.
8 D. I. CARGILL, *Analyst*, 87 (1962) 865.
E. STAHL, *Pharmazie*, 11 (1956) 633.

Received September 30th, 1963

Thin-layer chromatography of steroid conjugates

Various procedures have been reported for the chromatographic separation of steroid conjugates. For instance, group separation of steroid sulfates and glucuronosides may be achieved by adsorption chromatography on alumina[1,2], silica gel[3] and florisil[4] or by ion exchange chromatography on columns of DEAE or ECTEOLA cellulose and DEAE Sephadex[5]. Similar results can be obtained by paper chromatography, employing acidic or alkaline solvent systems on regular[6–10] or ion exchange paper[5]. In the present communication thin-layer chromatography of steroid sulfates and glucuronosides is described.

Thin layers of anion exchange cellulose (MN-300 G/DEAE and MN-300 G/ECTEOLA of Macherey, Nagel and Co., Düren, Germany) were prepared with regular equipment of Desaga, Heidelberg, Germany, using mixtures of 15 g DEAE cellulose (ion exchange capacity: 0.7 mequiv./g) or ECTEOLA cellulose (ion exchange capacity: 0.35 mequiv./g) in 80 and 75 ml water respectively. After 2 h at room temperature all plates were kept in an oven at 50° for 45 min. 25 μg of the different conjugates:

estrone sulfate*
dehydroepiandrosterone sulfate*
androsterone sulfate*
pregnenolone sulfate
17-hydroxypregnenolone sulfate
dehydroepiandrosterone glucuronoside**
androsterone glucuronoside**
etiocholanolone glucuronoside**,

dissolved in 0.01 ml methanol, were applied to the plates. The following solvent systems proved effective for adequate separation by ascending chromatography in an S-chamber:

(1) 0.5 *M* acetate buffer, pH 4.25
(2) 0.5 *M* acetate buffer, pH 4.75
(3) 1.0 *M* acetate buffer, pH 4.75
(4) 1.5 *M* acetate buffer, pH 5.00
(5) isopropanol–water–formic acid (65:33:2 v/v)
(6) ethanol–water–acetic acid (80:15:3 v/v)
(7) methanol–water–acetic acid (75:15:10 v/v).

While steroid conjugates with a 17-keto group were detected on dried (80°) plates by spraying with Zimmermann reagent, consisting of 2 vol. 2 % *m*-dinitrobenzene in 95 % ethanol and 1 vol. 15 % potassium hydroxide in 95 % ethanol, and subsequent heating to 75°, Δ^5-3β-hydroxysteroids were revealed by treatment with OERTEL-EIK-NES reagent[11], prepared from 1 vol. 95 % ethanol and 2 vol. conc. sulfuric acid.

All of the R_F values, given in Tables I and II, represent the mean from three chromatograms. As can be seen, satisfactory resolution of steroid sulfates and glucuronosides may be obtained by use of the solvent systems listed above. Lowering the pH or increasing the molarity of the buffer solution results in higher mobilities of steroid glucuronosides as well as steroid sulfates. Likewise, an increase in the acid concentration of the aqueous organic solvents leads to a rise in the mobility of steroid conjugates, especially pertaining to steroid glucuronosides, whereas the mobility of steroid sulfates is only slightly affected.

The application of thin-layer chromatography on anion exchange cellulose to the isolation of steroid conjugates in purified plasma extracts confirmed previous findings[11] for non-polar, solvolysable complexes, which differ markedly from authentic steroid sulfates, inasmuch as they remain at the origin of the thin-layer chromatograms.

* Kindly supplied by Prof. Dr. K. JUNKMANN, Schering AG, Berlin.
** Gift from Dr. A. E. KELLIE, Courtauld Institute of Biochemistry, Middlesex Hospital, London.

TABLE I

THIN-LAYER CHROMATOGRAPHY OF STEROID CONJUGATES ON DEAE CELLULOSE

Conjugate	*Rp value in solvent system*						
	1	*2*	*3*	*4*	*5*	*6*	*7*
Estrone sulfate	0.08	0.03	0.07	0.08	0.08	0.02	0.03
Dehydroepiandrosterone sulfate	0.19	0.09	0.14	0.20	0.22	0.07	0.06
Androsterone sulfate	0.23	0.15	0.22	0.24	0.33	0.08	0.07
Pregnenolone sulfate			0.27				
17-Hydroxypregnenolone sulfate			0.09				
Dehydroepiandrosterone glucuronoside	0.54	0.38	0.41	0.56	0.72	0.29	0.36
Androsterone glucuronoside	0.59	0.45	0.48	0.62	0.77	0.35	0.46
Etiocholanolone glucuronoside	0.56	0.40	0.45	0.58	0.75	0.31	0.39

TABLE II

THIN-LAYER CHROMATOGRAPHY OF STEROID CONJUGATES ON ECTEOLA CELLULOSE

Conjugate	*Rp value in solvent system*						
	1	*2*	*3*	*4*	*5*	*6*	*7*
Estrone sulfate	0.08	0.05	0.08	0.07	0.54	0.05	0.04
Dehydroepiandrosterone sulfate	0.16	0.14	0.23	0.15	0.79	0.11	0.08
Androsterone sulfate	0.23	0.18	0.29	0.21	0.85	0.13	0.09
Pregnenolone sulfate			0.34				
17-Hydroxypregnenolone sulfate			0.12				
Dehydroepiandrosterone glucuronoside	0.65	0.45	0.61	0.64	0.90	0.64	0.48
Androsterone glucuronoside	0.72	0.49	0.67	0.70	0.91	0.77	0.59
Etiocholanolone glucuronoside	0.67	0.47	0.64	0.65	0.91	0.71	0.55

Endokrinologische Abteilung, Institut für Hygiene und Mikrobiologie, Universität des Saarlandes,* Homburg/Saar (Germany)*

GEORG W. OERTEL
MARIA C. TORNERO
KÄTHE GROOT

1 J. J. BARLOW, *Biochem. J.*, 65 (1957) 34 P.
2 O. CRÉPY, M. F. JAYLE AND F. MESLIN, *Acta Endocrinol.*, 24 (1957) 233.
3 R. W. H. EDWARDS AND A. E. KELLIE, *Chem. Ind. (London)*, (1956) 250.
4 E. E. BAULIEU AND R. EMILIOZZI, *Bull. Soc. Chim. Biol.*, 44 (1961) 823.
5 G. W. OERTEL AND E. KAISER, *Biochem. Z.*, 336 (1962) 10.
6 I. E. BUSH, *Biochem. J.*, 67 (1957) 23 P.
7 I. E. BUSH AND M. M. GALE, *Biochem. J.*, 76 (1960) 10 P.
8 J. J. SCHNEIDER AND M. L. LEWBART, *J. Biol. Chem.*, 222 (1956) 787.
9 J. J. SCHNEIDER AND M. L. LEWBART, *Recent Progr. Hormone Res.*, 15 (1959) 201.
10 E. E. BAULIEU, *J. Clin. Endocrinol. Metab.*, 20 (1960) 900, 22 (1962) 501.
11 G. W. OERTEL AND K. B. EIK-NES, *Anal. Chem.*, 31 (1959) 98.
12 G. W. OERTEL, *Biochem. Z.*, 334 (1961) 431.

Received December 2nd, 1963

* Head: Prof. Dr. Dr. W. ZIMMERMANN.

Separation of 2,4-dinitrophenylosazones of vicinal dicarbonyls into classes by thin-layer chromatography

Several chromatographic methods have recently been reported for separation of 2,4-dinitrophenylosazones[1,2]. The method described by SCHWARTZ[2] provides a clue to carbonyl class by the color of developing bands on the partition column, but if the bands contain more than one class, it is difficult to ascertain the composition of such mixtures. The purpose of this communication is to describe a thin-layer chromatographic (TLC) procedure that affords separation of DNP-osazones into classes. Application of the procedure in conjunction with either of the aforementioned column partition procedures provides valuable clues to the identity of the DNP-osazones.

Two coatings were employed for preparing thin-layer plates: SeaSorb 43–Silica Gel G (1:1) and SeaSorb 43–Celite (anal. grade)–calcium sulfate (10:8.5:1.5). The mixtures were slurried with approximately 1.75 volumes of distilled water and coated onto plates (5 × 20 cm) in a 250 μ layer. The coated plates were dried in an oven at 110° for 2–12 h. Two solvent systems were used for separation of the DNP-osazones into classes: (a) benzene saturated with ethanolamine–8% methanol, and (b) chloroform–tetrahydrofuran–methanol (15:4:1). Development of the chromatograms was carried out in 600 ml beakers covered with a polyvinyl sheet, as described by LIBBEY AND DAY[3].

Fig. 1 illustrates movement of the DNP-osazones, using the benzene–ethanol-

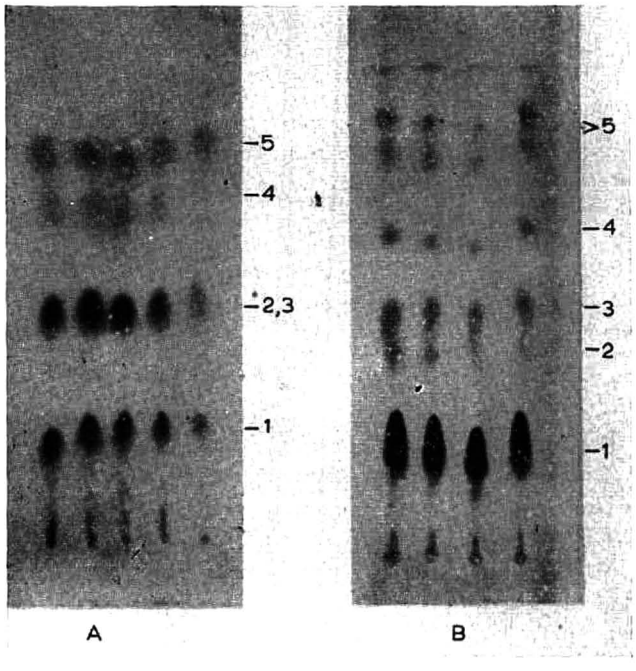


Fig. 1. Thin-layer chromatograms of DNP-osazones of vicinal dicarbonyls. Solvent: benzene saturated with ethanolamine–8% methanol. Plate A coated with SeaSorb 43–Silica Gel G (1:1); plate B coated with SeaSorb 43–Celite (anal. grade)–calcium sulfate (10:8.5:1.5). 1 = glyoxal; 2 = methylglyoxal; 3 = C_4–C_7 α-ketoalkanals; 4 = diacetyl; 5 = C_5 and C_7 2,3-diketones.

amine–methanol system. The first members of each dicarbonyl class behave differently from the remaining members of the homologous series. It will be noted that the osazones of diacetyl, glyoxal, and in certain instances, methylglyoxal, travel at a slower rate than the remaining homologs. Similar behavior has been reported on class separations of the DNP-hydrazones of monocarbonyls[4,5]. The colors of DNP-osazone spots on the TLC plates are indicative of their class: α-ketoalkanals exhibit blue color, glyoxal is blue to purple, and diketones are blue-green. The spots representing the vicinal dicarbonyl derivatives will fade after removal from the developing chamber, but the color can be intensified by spraying with 10% ethanolic KOH.

Freshly-prepared plates, dried 2 h in the oven, show maximum resolving power.

The only non-vicinal dicarbonyl osazone available in our laboratory was that of hexa-2,5-dione. This compound failed to migrate from the baseline when developed in the benzene solvent system.

This investigation was supported by P.H.S. research grant No. EF-00182 from the National Institutes of Health, Division of Environmental Engineering and Food Protection.

Department of Food Science and Technology, WILLIAM Y. COBB
Oregon State University, Corvallis, Oreg. (U.S.A.)

[1] E. A. CORBIN, *Anal. Chem.*, 34 (1962) 1244.
[2] D. P. SCHWARTZ, *J. Chromatog.*, 9 (1962) 187.
[3] L. M. LIBBEY AND E. A. DAY, *J. Chromatog.*, 14 (1964) 273.
[4] A. GADDIS AND R. ELLIS, *Anal. Chem.*, 31 (1959) 870.
[5] D. P. SCHWARTZ, O. W. PARKS AND M. KEENEY, *Anal. Chem.*, 34 (1962) 669.

Received October 15th, 1963

J. Chromatog., 14 (1964) 512–513

Dünnschicht-chromatographische Bestimmungen von Zuckern und Zuckeralkoholen auf Magnesiumsilikat

Magnesiumsilikat kann man nicht nur in der Säule[1], sondern auch auf Dünnschichtplatten[2] zur Chromatographie von Zuckern verwenden. Mit den Laufmitteln *n*-Propanol–Wasser–Chloroform (6:2:1) und *n*-Propanol–Wasser–Methyläthylketon (2:1:1) liess sich eine Reihe von Zuckern, Zuckeralkoholen und Glykolen gut identifizieren und trennen.

Die Untersuchungen wurden auf die Laufmittel *n*-Propanol–Wasser (5:5) und *n*-Propanol–Wasser–*n*-Propylamin (5:3:2) ausgedehnt. Tabelle I enthält die R_F-Werte, die unter Anwendung von je 2.5 γ bzw. bei nicht reduzierenden Zuckern und Zuckeralkoholen von je 5 γ in 0.001 ml wässriger Lösung ermittelt wurden.

Ersetzt man in dem Gemisch *n*-Propanol–Wasser (5:5) 40% des Wassers durch *n*-Propylamin, müsste man einen Abfall der R_F-Werte erwarten, da *n*-Propylamin in der eluotropen Reihe über dem Wasser stehen dürfte. Wie man der Tabelle I entnehmen kann, tritt der erwartete Abfall jedoch nur bei den untersuchten Ketosen,

TABELLE I

R_F-WERTE AUF MAGNESIUMSILIKAT WOELM ZUR DÜNNSCHICHT-CHROMATOGRAPHIE
(Platten lufttrocken, keine Kammersättigung)

Substanz	*n-Propanol–Wasser (5:5, V/V)*	*n-Propanol–Wasser–n-Propylamin (5:3:2, V/V)*	*Differenz*
L-Arabinose	0.61	0.65	+0.04
D-Arabinose	0.61	0.65	+0.04
D-Xylose	0.66	0.65	—0.01
D-Ribose	0.58	0.60	+0.02
L-Rhamnose	0.67	0.65	—0.02
D-Glucose	0.66	0.67	+0.01
D-Galactose	0.62	0.67	+0.05
D-Mannose	0.66	0.66	±0.00
Lactose	0.61	0.61	±0.00
Maltose	0.59	0.63	+0.04
D-Fructose	0.65	0.52	—0.13
L-Sorbose	0.66	0.55	—0.11
Saccharose	0.70	0.56	—0.14
Raffinose	0.64	0.43	—0.21
Trehalose	0.62	0.50	—0.12
Glycerin	0.74	0.61	—0.13
L-Arabit	0.70	0.55	—0.15
D-Sorbit	0.65	0.48	—0.17
D-Mannit	0.70	0.51	—0.19
Dulcit	0.67	0.50	—0.17

den nicht reduzierenden Zuckern und den Zuckeralkoholen ein. Bei den Aldosen beobachtet man im Gegenteil einen geringen Anstieg oder keine Änderung, höchstens nur einen geringen Abfall. Diese besitzen offenbar eine Affinität zum *n*-Propylamin. Hiermit in Übereinstimmung steht die Beobachtung, dass Procain, welches ebenfalls wie das *n*-Propylamin eine primäre Aminogruppe besitzt, auch nur mit Aldosen, nicht dagegen mit Ketosen Glykoside bildet[3,4], abgesehen von der Unmöglichkeit der Bildung bei nicht reduzierenden Zuckern und Zuckeralkoholen. Es handelt sich um die spontane Bildung in wässriger Lösung, während man unter gewissen Bedingungen auch Glykoside von Ketosen und von sekundären Aminen erhalten kann[5].

Aus Tabelle II ergibt sich, dass der beobachtete Effekt nur bei primären Aminen auftritt. Der R_F-Wert von Glucose liegt bei den primären Basen Isopropylamin, *n*-Propylamin und *n*-Butylamin höher als der R_F-Wert von Fructose. Bei dem sekundären Diäthylamin und den tertiären Basen Triäthylamin und Pyridin sind dagegen die R_F-Werte gleich.

Man hat die Möglichkeit, bei einem Zucker durch Chromatographie in den beiden Lösungsmittelgemischen der Tabelle I festzustellen, ob es sich um eine Aldose oder um eine Ketose bzw. nicht reduzierenden Zucker bzw. Zuckeralkohol handelt. Die Stoffe werden auf den Platten durch Besprühung mit einer 1 %igen wässrigen Kaliumpermanganatlösung sichtbar gemacht, sie erscheinen nach kurzer Zeit als helle Flecken auf dem violetten Untergrund. Hierbei brauchen die Platten bei Verwendung von *n*-Propanol–Wasser vor der Besprühung nur so lange an der Luft zu liegen, bis sie

TABELLE II

R_F-WERTE AUF MAGNESIUMSILIKAT WOELM ZUR DÜNNSCHICHT-CHROMATOGRAPHIE
(Laufmittel: *n*-Propanol–Wasser–Base (5:3:2, V/V); Platten lufttrocken, keine Kammersättigung)

	Isopropylamin	*n-Propylamin*	*n-Butylamin*	*Diäthylamin*	*Triäthylamin*	*Pyridin*
D-Glucose	0.75	0.67	0.60	0.43	0.32	0.45
D-Fructose	0.55	0.52	0.46	0.43	0.33	0.44

äusserlich trocken erscheinen. Bei Verwendung von *n*-Propanol–Wasser–*n*-Propylamin muss man sie erst 2 Std. an der Luft trocknen und dann noch 1 Std. bei 130° im Trockenschrank erhitzen, um das *n*-Propylamin restlos zu entfernen, das die violette Färbung der Platten schnell in eine braune umschlagen lässt. Es sollten nur reinste Lösungsmittel verwendet werden, da sonst, besonders bei dem *n*-Propylamin haltigen Gemisch, unscharfe Flecken entstehen können. Die Herstellung der Platten erfolgte mit einem Streichgerät.

Während der Trocknung der Platten bei 130° erscheinen die Aldosen und Ketosen als schwache braune Flecken, nicht dagegen die nicht reduzierenden Zucker und die Zuckeralkohole. Diese Unterscheidung von den Ketosen, die sich nicht aus dem Verhalten der R_F-Werte ergibt, lässt sich noch durch eine andere Farbreaktion erreichen: Besprüht man die trocknen Platten mit einer Lösung von 1.7 g Diphenylamin in 75 ml mit Wasser gesättigtem *n*-Butanol, trocknet dann an der Luft und anschliessend 20 Min. bei 130°, geben die Aldosen und Ketosen kräftige blaue Flecken, während die nicht reduzierenden Zucker und die Zuckeralkohole diese Farbreaktion nicht zeigen. Bei den reduzierenden Disacchariden Lactose und Maltose sind die blauen Flecken nur angedeutet, auch bei Mengen von 10 γ.

Mit dem Lösungsmittelgemisch *n*-Propanol–Wasser–*n*-Propylamin (5:3:2) lassen sich ausgezeichnete Trennungen vornehmen, insofern in den Gemischen die zu trennenden Verbindungen verschiedenen Gruppen angehören, also Glucose und Saccharose, Mannose und Mannit, Galactose und Dulcit, Arabinose und Arabit, Glucose und Sorbit. Glucose und Fructose lassen sich besser mit *n*-Propanol–Wasser–Isopropylamin (5:3:2) trennen, bei Sorbose und Sorbit muss man als Laufmittel *n*-Propanol–Wasser (17.5:2.5) verwenden (R_F-Werte 0.21 bzw. 0.11).

Chemisches Laboratorium der Fa. M. Woelm, Eschwege (Deutschland) HERBERT GRASSHOF

1 B. W. LEW, M. L. WOLFROM UND R. M. GOEPP, *J. Am. Chem. Soc.*, 68 (1946) 1449.
2 H. GRASSHOF, *Deut. Apotheker-Ztg.*, 103 (1963) 1396.
3 J. S. CANNEL, *Pharm. J.*, 167 (1951) 231.
4 H. GRASSHOF UND U. LIPPOLD, *Arzneimittel-Forsch.*, 3 (1953) 42.
5 R. KUHN UND L. BIRKOFER, *Ber.*, 71 (1938) 621.

Eingegangen den 25. November 1963

Separation of ^{131}I-labelled monoiodotyrosine and diiodotyrosine by thin-layer chromatography

In the course of work on the preparation of iodinated tyrosines labelled with ^{131}I at very high specific activity, it was necessary to find a method for separating ^{131}I-labelled monoiodotyrosine (MIT) from ^{131}I-labelled diiodotyrosine (DIT).

When iodinated tyrosines are prepared, the separation of ^{131}I-MIT, ^{131}I-DIT, iodide ions and unreacted tyrosine may be performed by paper chromatography, according to the method described by LEMMON *et al.*[1].

In order to carry out a faster separation on a preparative scale of MIT and DIT, we have developed a thin-layer chromatographic method.

Thin-layer plates (5 × 20 cm) were prepared in the usual way with silicagel "G" Merck, using the Desaga apparatus, and dried at 120° for 20 min. Several elution mixtures were employed and the results are reported in Table I.

TABLE I

R_F VALUES FOR MIXTURES OF TYROSINE, IODINATED TYROSINES AND IODIDE IONS

Solvents: a = phenol–water (75:15); development time = 7 h; b = *n*-butanol–acetic acid–water (10:1:1); development time = 5 h; c = *n*-butanol–acetic acid–water (4:1:5); development time = 5 h; d = *n*-butanol–methanol–NH_4OH, 20% (8:2:2); development time = 6 h.

Substance	$R_F \times 10^2$			
	a	*b*	*c*	*d*
MIT	52	41	64	54
DIT	77	56	75	45
Tyrosine	40	23	36	26
I^-	14	70	28	27

The spots of ^{131}I-labelled MIT, DIT, and I^- were identified by scanning the plate. The scanning apparatus consists of a mechanical device, which causes the silica gel plate to slide under a collimated thin-window G. M. counter. The chromatographic pattern is obtained by means of a ratemeter–recorder system.

The tyrosine spot was identified by spraying a 0.2% ninhydrin solution in dry acetone.

Standards of pure ^{131}I-labelled MIT and DIT were prepared by the method of LEMMON *et al.*[1].

The chromatographic separation method described above may be usefully employed to isolate ^{131}I-labelled MIT and DIT on a preparative scale. The iodinated mixture (0.4 ml) containing ^{131}I-MIT (50 μg), ^{131}I-DIT (50 μg), I^- (30 μg) and unreacted tyrosine (100 μg) are spotted on the plate at a distance of about 2 cm from the edge.

n-Butanol–acetic acid–water (4:1:5) is used as elution mixture (see Fig. 1). After development (5 h), the spots corresponding to MIT and DIT are removed from the plate. The silica gel is then collected in centrifuge tubes, and 0.1 *N* NH_4OH in 10% isopropanol solution (0.4 ml) is added. The mixture is shaken for a short time and centrifuged, and the supernatant liquid is pipetted into a small test tube. After three extractions, about 90% of both MIT and DIT is recovered.

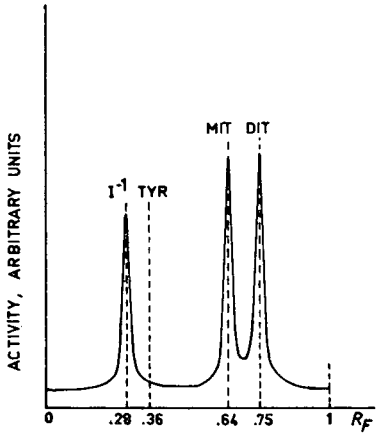


Fig. 1. Thin-layer radiochromatography of a mixture of MIT, DIT, I^-, eluted with *n*-butanol–acetic acid–water (4:1:5).

The yield was evaluated by counting of the supernatant.

When compared with separation methods on paper, thin-layer chromatography has the advantage of reducing the operation time, making this technique suitable for routine controls in the production of labelled MIT and DIT.

In addition, the higher adsorption capacity of silica gel in plate chromatography is very useful for preparative purposes.

SORIN, Centro Ricerche Nucleari,
Saluggia, Vercelli (Italy)

A. MASSAGLIA
U. ROSA

[1] R. M. LEMMON, W. TARPEY AND K. G. SCOTT, *J. Am. Chem. Soc.*, 72 (1950) 758.

Received October 14th, 1963

Thin-layer chromatography of metabolic derivatives of tryptophan

Paper chromatography is routinely used in our laboratory for the identification and determination of several metabolic derivatives of tryptophan of the so-called "via kynurenine", in urine of normal and pathological subjects. This method, which is suitable for quantitative analyses, is, however, time-consuming when used for qualitative purposes[1].

Therefore, an attempt was made to apply thin-layer chromatography (TLC) to the separation of tryptophan metabolites. The technique described here not only has the usual advantages of TLC, but several others as well, particularly as regards speed, in which it surpasses the previous method.

DIAMANTSTEIN AND EHRHART[2] were the first to apply TLC on silica gel to the fractionation of tryptophan, indole, indican, anthranilic and quinolinic acids. We

achieved more reproducible results than the above-mentioned authors by employing polyamide as an adsorbent for the chromatography of compounds derived from tryptophan.

Polyamide has already been used for the isolation of phenolic substances, since it has a high adsorption capacity and, moreover, the sorption processes are reversible, which is useful for analytical purposes. Thin layers of polyamide have been employed for the separation of flavanoids[3–5], polyhydroxyphenols and pro-anthocyanidins[6], antioxidants[7], gallic acid and its esters[8], and essential oils[9].

This new technique on thin layers of polyamide powder has some advantages, particularly as regards resolution power, in comparison with that on silica gel; polyamide powder is also undoubtedly preferable to other materials tried by us, such as dextran, neutral, basic or acid alumina and kieselguhr.

Table I reports the results obtained with polyamide as an adsorbent, of a rapid chromatographic separation of nine tryptophan derivatives. The best results are obtained when the mixture formic acid–methanol–water (2.5:37.5:60) is employed.

Substitution of acetic acid for formic acid, as well as the use of higher alcohols instead of methanol causes a decrease in the quality of fractionation.

TABLE I

R_F VALUES AND FLUORESCENCES

Adsorbent: polyamide (Woelm).
Solvent: 99% formic acid–methanol–water (2.5:37.5:60)

Compound	*Mean R_F*	*Fluorescence at 3655 Å*
Xanthurenic acid	0.17	blue-green
Xanthurenic acid 8-methyl ether	0.30	blue
Kynurenic acid	0.36	blue (turning yellowish)
3-Hydroxyanthranilic acid	0.50	blue-violet
N-α-Acetyl-3-hydroxykynurenine	0.68	yellow-green
o-Aminohippuric acid	0.73	violet
N-α-Acetylkynurenine	0.78	azure
3-Hydroxykynurenine	0.86	yellow-green
Kynurenine	0.90	azure

When methyl, ethyl, propyl, isopropyl, butyl and isobutyl alcohols and ethylene and propylene glycols mixed with water in different ratios are employed negative results are obtained. Mixtures such as pyridine–*n*-butanol–water, triethanolamine–*n*-butanol–water, pyridine–isopropanol–water, ammonia–isopropanol–water, pyridine–methanol, and ammonia–methanol in various ratios give front and spot splitting and have a detrimental effect on the compounds chromatographed. Large and overlapping spots result when 0.1, 1, 3 and 5% aqueous solutions of NaCl, KCl, Na_2HPO_4, NaH_2PO_4, $NaNO_3$, Na_2SO_4 and CH_3COONa are used.

When urine is chromatographed directly no R_F variation is observed. By running standard solutions containing pure compounds on the same chromatoplate the spots can be identified and at the same time a rough quantitative comparison between known and unknown samples can be made.

Since, however, the volumes of urine to be spotted are very small (0.001–0.05 ml), the method is only suitable when the urinary amounts of metabolites are above the normal levels of "spontaneous" excretion.

Experimental

Materials. Kynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, xanthurenic acid 8-methyl ether, and *o*-aminohippuric acid were prepared in this laboratory. N-α-Acetylkynurenine and N-α-acetyl-3-hydroxykynurenine, chromatographically pure, were isolated from human urine of subjects loaded with L-tryptophan. 3-Hydroxyanthranilic acid was a commercial product. Urine of human beings who had been subjected to a loading test of 100 mg/kg of L-tryptophan was also analysed, the content of the above metabolites being markedly elevated in this case. The polyamide powder was the standardized product of Woelm.

Solvent systems. Of the many solvents tried, formic acid 99%–methanol–water (2.5:37.5:60 v/v), gave the best results.

Detection of the spots. For detecting the above tryptophan derivatives, a Philips HPW-125 lamp at 3655 Å, was used.

Method. Smooth glass strips (4 × 20 or 4 × 30 cm) were coated with a 0.1 mm thick layer of standardized polyamide (0.3 g suspended in a mixture of methanol–chloroform, 3:2), by means of a home-made applicator. The plates were then dried in an oven at 40° for 10 min.

Spots of the standard solutions of the above compounds in 50% acetone were applied from a capillary pipette on a point 2.5 cm from the edge of the plate. Amounts of each metabolite ranging between 0.05–3 μg gave good resolutions.

Urine of subjects loaded with L-tryptophan was directly spotted in volumes of 0.001–0.05 ml, depending upon the content of metabolites, and a mixture of pure substances was run in parallel on the same chromatostrips.

Development was carried out by the ascending method in closed cylindrical chambers (6 × 40 cm) in which saturation was not necessary. The time required for the development of short and long chromatostrips (the solvent front moved 15 and 26 cm) was 60 and 150 min, respectively.

The developed chromatograms were then removed from the tanks, dried in an oven at 60° and observed under U.V. light.

Institute of Pharmaceutical Chemistry,
University of Padova, Padova (Italy)

C. A. BENASSI
F. M. VERONESE
E. GINI

[1] D. COPPINI, C. A. BENASSI AND M. MONTORSI, *Clin. Chem.*, 5 (1959) 391.
[2] T. DIAMANTSTEIN AND H. EHRHART, *Z. Physiol. Chem.*, 326 (1961) 131.
[3] J. DAVÍDEK AND E. DAVÍDKOVÁ, *Pharmazie*, 16 (1961) 352.
[4] K. EGGER, *Z. Anal. Chem.*, 182 (1961) 161.
[5] L. BIRKOFER, C. KAISER, H. A. MEYER-STOLL AND F. SUPPAN, *Z. Naturforsch.*, 17b (1962) 352.
[6] W. MOHR, *Fette, Seifen, Anstrichmittel*, 64 (1962) 831.
[7] J. DAVÍDEK AND J. POKORNÝ, *Z. Lebensm.-Untersuch.-Forsch.*, 115 (1961) 113.
[8] J. DAVÍDEK, *J. Chromatog.*, 9 (1962) 363.
[9] YAO-TANG LIN, KUANG-TSAN WANG AND YAU-SAN LIN, *J. Chinese Chem. Soc. (Taiwan)*, 9 (1962) 68.

Received January 27th, 1964

A simplified method for the quantitative determination of pregnanediol in urine

For many years the determination of pregnanediol (5β-pregnane-3α,20α-diol) has been used to estimate corpus luteum function, pregnanediol being the most important metabolite of the corpus luteum hormone. The methods employed usually comprise the following steps: (1) hydrolysis of the pregnanediol glucuronide, the form in which most of the pregnanediol is excreted in the urine, (2) extraction of the free pregnanediol, (3) separation of the pregnanediol from other compounds simultaneously extracted and (4) some sort of quantitative (or semiquantitative) estimation of the separated pregnanediol.

In some of the early methods, step (3) was omitted and the extract was estimated gravimetrically. As the extract from the urine contains many different compounds (including other closely related corpus luteum metabolites) this method was not exact. The methods were greatly improved when separation of the extracted compounds was carried out by column chromatography (KLOPPER, MICHIE AND BROWN[1]). Since elution of pregnanediol from the column without being contaminated by other compounds, especially the other chemically related corpus luteum hormone metabolites, is very difficult, it was found to be more advantageous to carry out the separation by thin-layer chromatography (TLC). By this method it is possible to localize the pregnanediol spot precisely and separate it from any other compound. WALDI[2] has developed a method using this principle, which gives a semiquantitative estimation of the amount of pregnanediol in urine and proposes the method as a pregnancy test. STARKA AND MALÍKOVÁ[3] have described a method for the quantitative assay of urine pregnanetriols using TLC followed by elution of the pregnanetriols from the spot and a colorimetric estimation of the eluted pregnanetriols with sulphuric acid as described by FOTHERBY AND LOVE[4].

The TLC method may be simplified by carrying out the sulphuric acid reaction directly on the material scraped from the plate. In this way it is possible to make a quantitative estimation of any of the urine pregnanediols and pregnanetriols, both in pregnancy urine and non-pregnancy urine from any part of the menstrual cycle.

Procedure

Hydrolysis with hydrochloric acid and extraction with cyclohexane is carried out according to WALDI[2]. 100 ml urine or less is used depending on the pregnanediol content of the urine.

The extract is evaporated to dryness and the residue dissolved in $^1/_2$ ml chloroform. 50 μl of the solution is chromatographed on silicagel with chloroform–acetone (90:10) as solvent. Simultaneously, on the same plate standards (*e.g.* 30 and 50 μl) of a 0.1 % (w/v) solution of pure pregnanediol in chloroform are run. After the solvent front has moved 10 cm (in an S-chamber[5]), the plate is dried and sprayed with distilled water. The pregnanediols are visible as white spots on a grey background. The urinary pregnane-3α,20α-diol is localized by means of the R_F values of the standards. The spots of pregnanediol are marked with a pencil point and the plate is dried. When dry, the area of the layer containing the pregnanediol is scraped off and the material introduced into a glass tube; 3 ml concentrated sulphuric acid is

added and mixed with the material. After standing for 10 min at room temperature and centrifuging, the optical density of the supernatant is measured in a spectrophotometer at 430 mμ. A blank is prepared from a similar area of the silicagel layer as the spots, but without any pregnanediol or evidently coloured compounds on it. The spots of the standard solution of pregnanediol are treated in the same way as the urinary extracts. With the optical density as ordinate and the concentration of pregnanediol as abscissa a curve is drawn which should be rectilinear. The content of pregnanediol in the spots from the urinary extracts is estimated by means of this standard curve. The whole procedure from hydrolysis to photometry may be carried out on 2–3 urine specimens (in triplicate) by one technician in about 6 hours.

Discussion

The ability of TLC to separate compounds chemically closely related to pregnanediol from the latter is illustrated in Table I, where the R_F values of the compounds examined are given, and in Fig. 1 the TLC of the compounds is shown.

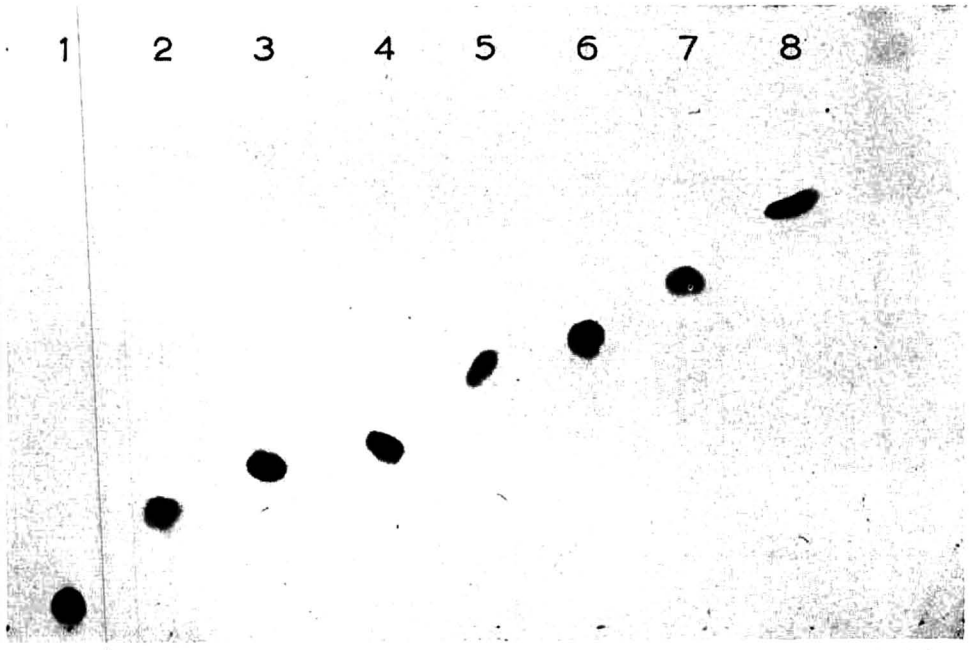


Fig. 1. Steroids related to pregnanediol. Solvent: chloroform–acetone 90:10 on silicagel. Colour development on the plate with sulphuric acid and by heating. For the compounds, see Table I.

The effect of time on the colour development with concentrated sulphuric acid was investigated (Fig. 2) and it was found that the colour development was complete after 10 min and did not change measurably during 40 min; Fig. 3 shows that with a development time of 10 min the Lambert-Beer law was obeyed.

At a level of 1–4 mg/l urine the results are expressed as the mean of duplicate determinations ±0.2 mg, and the 95 % confidence limits calculated from duplicate determinations on 18 urine samples were 0.14 mg.

TABLE I

THE R_F VALUES OF VARIOUS STEROIDS RELATED TO PREGNANEDIOL

	Steroid	*R_F values*
1	5β-Pregnane-3α,6α-diol-20-one	0.03
2	Pregnanediol (5β-pregnane-3α,20α-diol)	0.17
3	5α-Pregnane-3β,20α-diol	0.26
4	5α-Pregnane-3β,20β-diol	0.29
5	5β-Pregnan-3α-ol-20-one	0.39
6	5α-Pregnan-3β-ol-20-one	0.43
7	5β-Pregnan-3β-ol-20-one	0.55
8	5β-Pregnane-3,20-dione	0.65

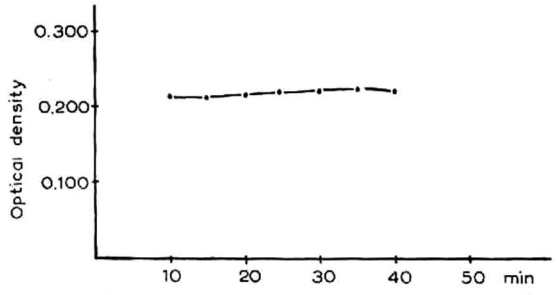


Fig. 2. Colour development with conc. sulphuric acid on the plate. Optical density as ordinate related to time in minutes as abscissa.

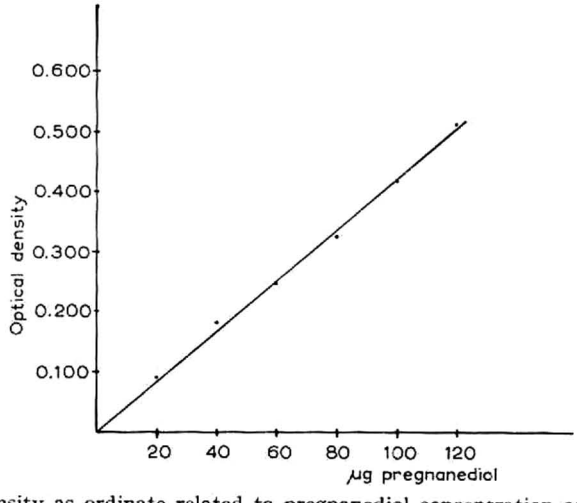


Fig. 3. Optical density as ordinate related to pregnanediol concentration as abscissa. Reaction with concentrated sulphuric acid for 10 min.

The content of pregnanediol in the morning urines of fertile, non-pregnant women was found to be about 1 mg/1000 ml in the pre-ovulatory phase and about 6 mg/1000 ml in the late post-ovulatory phase.

This study was carried out with the technical assistance of Mrs. ELLA ANDERSEN and Miss LATRELLE HASTINGS.

Biochemical Department of the Municipal Hospital, Aalborg (Denmark) H. O. BANG

[1] A. KLOPPER, E. A. MICHIE AND J. K. BROWN, *J. Endocrinol.*, 12 (1955) 209.
[2] D. WALDI, *Klin. Wochschr.*, 16 (1962) 827.
[3] L. STARKA AND J. MALÍKOVÁ, *J. Endocrinol.*, 22 (1961) 215.
[4] K. FOTHERBY AND D. N. LOVE, *J. Endocrinol.*, 20 (1960) 157.
[5] E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, 1962.

Received December 19th, 1963

J. Chromatog., 14 (1964) 520–523

Dünnschichtchromatographische Trennung von Ketocarbonsäuren

Prinzip

Die Ketocarbonsäuren wurden mit 4-Oxo-2-thion-thiazolidin (Rhodanin) in die entsprechenden Rhodaninderivate übergeführt, die sich auf Acetylcellulose-Schichten trennen lassen. Untersucht wurden (angeordnet nach steigender Zahl der Kohlenstoffatome), die in Tabelle I angegebenen Ketocarbonsäuren.

TABELLE I

R_F-WERTE DER RHODANINDERIVATE DER KETOCARBONSÄUREN IN VERSCHIEDENEN ELUTIONSMITTELN

	R_F			
	I	*II*	*III*	*IV*
α-Ketopropionsäure	0.55	0.22	0.32	0.51
Oxalessigsäure	0.54	0.22	0.32	0.51
α-Ketobuttersäure	0.67	0.34	0.44	0.59
α-Ketoisovaleriansäure	0.71	0.47	0.53	0.65
α-Keto-*n*-valeriansäure	0.73	0.51	0.57	0.70
γ-Keto-*n*-valeriansäure	0.59	0.26	0.41	0.54
α-Ketoglutarsäure	0.29	0.04	0.09	0.27
α-Ketocapronsäure	0.78	0.65	0.68	0.78
α-Keto-D-gluconsäure, Kaliumsalz	0.68	—	0.58	0.67
α-Ketoisocapronsäure	0.78	0.70	0.67	0.77
α-Ketooenanthsäure	0.83	0.79	0.77	0.85
α-Ketocaprylsäure	0.87	0.83	0.85	0.91
Phenylglyoxylsäure	0.65	0.54	0.47	0.59
α-Ketopelargonsäure	0.74	0.62	0.50	0.75
α-Ketophenylpropionsäure	0.60	0.47	0.66	0.65
4-Hydroxyphenylbrenztraubensäure	0.67	0.42	0.49	0.71
Reagenz			0.60	0.70

Reagenzlösung

250 mg Rhodanin, 5 Tropfen Ammoniakflüssigkeit (25 %ig) und 50 mg Ammoniumchlorid werden in 100 ml Äthanol unter leichtem Erwärmen gelöst.

Lösung der Ketosäuren

0.5 %ig in Äthanol (96 %ig). Das Kaliumsalz der 2-Keto-D-gluconsäure wird 0.5 %ig in einem Gemisch aus gleichen Volumteilen Äthanol 96 %ig–Wasser gelöst.

Herstellung der Rhodanin-Derivate

2.0 ml der jeweilig gelösten Ketosäure werden mit 4.0 ml der erkalteten Reagenzlösung 10 Min. lang im Wasserbad unter Rückfluss bei 65–75° erwärmt.

Herstellung der Sorptionsschicht

(Ausreichend für 5 Platten 200 × 200 mm.) 10.0 g Cellulosepulver MN 300 Ac werden mit 50 ml Methanol und 5 ml Wasser gemischt und anschliessend im Starmix 1–2 Min. lang homogenisiert. Die beschichteten Platten werden 5–10 Min. lang bei 60° getrocknet.

Chromatographie

Elutionsmittel. I. *n*-Propanol–*n*-Butanol–Ammonkarbonatlösung* (40:20:30, v/v); II. *n*-Propanol–*n*-Butanol–Ammonkarbonatlösung (30:30:30, v/v); III. *n*-Propanol–*n*-Butanol–Ammonkarbonatlösung (35:25:30, v/v); IV. *n*-Propanol–Ammonkarbonatlösung (2:1) v/v).

Trennstrecke: 100 mm; Aufgetragene Menge: 0.5–2 γ. Laufzeit: Je nach Art des angewandten Elutionsgemisches 100–120 Min.

Chromatographiert wird bei Zimmertemperatur unter Kammerübersättigung.

Detektion

Die Reaktionsprodukte der Ketosäuren sind im U.V.-Licht bei 365 mμ als dunkle Flecken zu erkennen, die überschüssige Reagenzlösung bei 254 mμ.

Pharmazeutisches Institut der Universität Bonn, Bonn (Deutschland)

MELANIE RINK
SIGRID HERRMANN

Eingegangen den 7. Oktober 1963

* Die Ammonkarbonatlösung besteht aus zwei Teilen 10%iger wässriger Ammonkarbonatlösung und einem Teil 5 *N* Ammoniak.

Determination of triglyceride composition by horizontal thin-layer chromatography

Since thin-layer chromatography was developed[1] into a simple and reliable analytical technique, it has proved to be extremely useful in many fields of chemistry. In the chemistry of fats, for instance, the technique enables 100 mg amounts of complicated triglyceride mixtures to be separated and analysed. This has been achieved by using 1 mm thick layers of silica gel impregnated with silver nitrate, on 20 × 40 cm glass plates, which were covered by another plate in order to ensure the immediate saturation of the air space so enclosed with solvent vapour[2]. The preparation of the $AgNO_3$–silica layers has been described elsewhere[3]; the apparatus was in essence identical to that described by BRENNER *et al.*[2], but adapted to the dimensions of the 20 × 40 cm glass plates.

100 mg triglyceride mixture, dissolved in *ca.* 1 ml light petroleum or carbon tetrachloride, was brought onto the adsorbent layer in a 16 cm long streak at a distance of *ca.* 4 cm from one of the short edges of the plate, and then developed with a suitable solvent mixture. Using this technique, the formation of droplets on the cover is generally observed[4], but this could easily be prevented by covering the apparatus with a light-excluding cloth. After *ca.* 4 h the solvent front had run a distance of 35 cm. A good, visual indication of the progress of the separation during the chromatographic process was obtained by the introduction of small amounts of suitable dyes at the start. Decanal-DNPH behaves as glyceryl tristearate (SSS)* while Sudan-III (ex Fluka, Switzerland) contains two dyes that behave as triglycerides with 1 and 2 *cis* double bonds respectively. When the SSS fraction had reached the top of the plate the process was stopped and the fractions then obtained[3,5] were examined by various techniques. Very good results have thus been obtained.

The following mixtures have been quantitatively analysed:

1. *An interesterified mixture of equal amounts of glyceryl tristearate (SSS), trielaidate (EEE) and trioleate (OOO)*, in which the acyl groups are expected to be randomly distributed. Apart from positional isomers of the type SOO, OSO etc., in which the radicals are in a different position, ten different triglycerides are expected to be present in this mixture. Using benzene as eluant, this mixture was well separated into 7 fractions. The results are compiled in Table I.

Recoveries were at least 97 %. The fatty acid composition of each fraction was used for identification, and was found to be in good agreement with the theoretical values.

2. *Natural fats.* Palm oil was easily separated into five fractions, and soyabean oil into seven fractions, using as eluants benzene–diethyl ether (95:5 and 85:15 v/v respectively). In view of the results of experiments with synthetic triglycerides[5,6] using column chromatography on silica impregnated with silver nitrate, each fraction was assumed to contain only triglycerides of the same degree of unsaturation. The triglyceride composition of each fraction was further calculated from the total fatty acid composition determined by G.L.C. and from the fatty acid composition at the 2-positions determined by semi-micro lipase splitting[7]. The results allowed of complete and accurate triglyceride analysis of these very complicated fats. As one of the most

* S = stearate; O = oleate; E = elaidate.

TABLE I

TRIGLYCERIDE COMPOSITION OF INTERESTERIFIED SSS, EEE AND OOO

Fraction No.	Content	Amount (%)		Distance from start (cm)	
		Found	Calc.	to lower side of fraction band	to upper side of fraction band
1	OOO	5.6	3.7	2.2	4.5
2	EOO	9.7	11.1	5.1	7.0
3	SOO + EEO	20.7	22.2	7.9	11.5
4	EEE + SEO	25.6	25.9	13.0	18.5
5	SSO + SEE	21.4	22.2	19.7	24.6
6	SSE	11.8	11.1	25.6	30.0
7	SSS	5.1	3.7	33.0	36.0

important results the correctness of VANDER WAL's distribution theory[8] was ascertained.

Other synthetic mixtures as well as natural and hardened fats are currently being analysed. Details of the results and of the experimental conditions will be published in due course.

Unilever Research Laboratory,
Vlaardingen (The Netherlands)

B. DE VRIES*
G. JURRIENS

[1] E. STAHL (Editor), *Dünnschichtchromatographie*, Springer-Verlag, Berlin, 1962.
[2] M. BRENNER AND A. NIEDERWIESER, *Experientia*, 17 (1961) 237, *cf.* Ref. 1, p. 24.
[3] B. DE VRIES AND G. JURRIENS, *Fette, Seifen, Anstrichmittel*, 65 (1963) 725.
[4] P. M. REISTERT AND D. M. SCHUMACHER, *Experientia*, 19 (1963) 84.
[5] G. JURRIENS, B. DE VRIES AND L. SCHOUTEN, *J. Lipid Res.*, in press.
[6] B. DE VRIES, *J. Am. Oil Chemists' Soc.*, in press.
[7] G. JURRIENS, B. DE VRIES AND L. SCHOUTEN, *J. Lipid Res.*, in press.
[8] R. J. VANDER WAL, *J. Am. Oil Chemists' Soc.*, 37 (1960) 18.

Received September 24th, 1963

* Present address: Unilever Research Laboratory, Duiven (The Netherlands).

Beitrag zur Dünnschichtchromatographie von Barbituraten*

In einer früheren Arbeit beschäftigten wir uns mit der adsorptionschromatographischen Trennung von Barbituraten unter Verwendung von Aluminiumoxyd-Säulen[1]. Inzwischen hat die Dünnschichtchromatographie vor allem durch die Untersuchungen von STAHL[2] eine starke Verbreitung erfahren. So wurde diese elegante Schnellmethode schon verschiedentlich für die Identifizierung von Barbituraten herangezogen[3–8].

* Herrn Prof. Dr. ADOLF BÜRGIN (Bern) zum 60. Geburtstag gewidmet.

Wir stellten uns nun die Aufgabe, zu untersuchen, inwieweit sich die wichtigsten in pharmazeutischen Zubereitungen anzutreffenden Barbituratkombinationen dünnschichtchromatographisch auftrennen lassen. Es ging uns also nicht darum, ein Laufmittel zu finden, welches eine vollständige Trennung sämtlicher Barbiturate erlauben würde. Dieses Ziel dürfte nach unserer Auffassung kaum zu erreichen sein. Um möglichst kurze Trennzeiten erzielen zu können, wurden für unsere Untersuchungen drei *wasserfreie* Fliessmittel mit folgender Zusammensetzung ausgewählt.

I. Benzin–Dioxan (5:2) von KLÖCKING[9] für die papierchromatographische Trennung von Barbituraten mit Dimethylformamid als stationäre Phase benutzt.

II. Chloroform–Aceton (9:1) nach BÄUMLER UND RIPPSTEIN[4].

III. Benzol–Äther (1:1).

Methodisches

Fliessmittel. Für die Herstellung der Fliessmittel wurden folgende Lösungsmittel-Qualitäten verwendet:

Aceton rein, Siegfried AG.
Äther für Narkose, Siegfried AG.
Benzol PhHV
Benzin PhHV
Chloroform PhHV
Dioxan für Chromatographie, Merck AG.
Das Fliessmittel wurde für jede Platte frisch angesetzt.

Herstellung der Platten. Es wurde die Grundausrüstung nach STAHL (Fa. Desaga, Heidelberg) benützt. 30 g Kieselgel G (Merck) wurden mit 60 ml Wasser während 1 Min. in einem Erlenmeyerkolben mit Schliff kräftig geschüttelt und das Gemisch auf 20 × 20 cm Glasplatten aufgetragen (Schichtdicke 275 μ). Die Platten wurden anschliessend 15 Min. an der Luft bei Zimmertemperatur, dann während 30 Min. in einem auf 110° eingestellten Trockenschrank mit Luftumwälzung getrocknet und vor der Benützung 16–20 Std. in einem Exsiccator über Blaugel aufbewahrt.

Auftragen der Substanzen. Je 10 mg Substanz wurden in 2 ml Aceton gelöst und 4 μl dieser Lösung, entsprechend 20 γ, auf die Startpunkte aufgebracht.

Chromatographie. Gearbeitet wurde mit Kammersättigung. Zu diesem Zweck kleidete man die Trennkammern mit Filtrierpapier aus, welches in das Fliessmittel eintauchte. Letzteres wurde 15–20 Min. vor der Chromatographie eingefüllt.

Temperatur: 20°.
Laufstrecke: 10 cm (Frontlinie durchgehend markiert).
Zeit: Fliessmittel I und II, 20–25 Min.
Fliessmittel III, 15–20 Min.

Sichtbarmachung. Nach der Chromatographie wurden die Platten ca. 10 Min. an der Luft trocknen gelassen und mit einer 1 %igen wässerigen Hg(I)-nitratlösung vorsichtig besprüht, bis zum Erscheinen der weissen bezw. grauen oder schwarzen Flecke.

Ergebnisse

In Tabelle I sind die durchschnittlichen R_F-Werte der einzeln untersuchten Barbiturate und Hydantoine zusammengestellt. Die Abweichungen der Einzelwerte vom

TABELLE I

R_F-WERTE DER EINZELN UNTERSUCHTEN BARBITURATE UND HYDANTOINE

Nr.	Substanz	Mittlere R_F-Werte in Fliessmittel			Farbe nach Besprühen mit Hg(I)-nitrat	Nachweisgrenze (γ)
		I	II	III		
1	Allobarbital	0.33	0.32	0.54	schwarz	1
2	Amobarbital	0.40	0.38	0.55	grau	2.5
3	Aprobarbital	0.38	0.31	0.55	schwarz	2.5
4	Barbital	0.34	0.24	0.45	schwarz	1
5	Butabarbital	0.37	0.32	0.50	grau	2.5
6	Cyclobarbital	0.36	0.32	0.50	grau	2.5
7	Cyclopal®	0.34	0.36	0.55	weiss	2.5
8	Dipropylbarbitursäure	0.41	0.36	0.55	grau	1
9	Hexobarbital	0.41	0.46	0.54	grau-schwarz	2.5
10	Methylphenobarbital	0.38	0.53	0.56	weiss	2.5
11	Pentobarbital	0.40	0.36	0.55	grau	2.5
12	Phenobarbital	0.27	0.25	0.49	weiss	5
13	Sandoptal®	0.39	0.38	0.57	grau-schwarz	1
14	Secobarbital	0.39	0.41	0.59	grau	2.5
15	Thiopental	0.52	0.68	0.69	schwarz	2.5
16	Diphenylhydantoin	0.16	0.19	0.33	weiss	10
17	Methylphenyläthylhydantoin	0.29	0.43	0.35	weiss	10
18	Phenyldibromäthylmethyl-hydantoin*	0.15	0.16	0.21	weiss	10

* 5-Methyl-5-(1,2-dibrom-2-phenyl-äthyl)-hydantoin.

Mittelwert betrugen im allgemeinen ± 0.02, in Ausnahmefällen ± 0.05. Es ist ersichtlich, dass das Fliessmittel III die schlechteste Differenzierung ergibt. Die Nachweisgrenzen liegen bei den Barbituraten (mit Ausnahme des Phenobarbitals) bei 1 bezw. 2.5 γ, bei den untersuchten Hydantoinen bei 10 γ.

Tabelle II enthält die Resultate der Trennungen in der Praxis vorkommender Barbiturat-Gemische. Sämtliche Kombinationen liessen sich in befriedigender Weise

TABELLE II

TRENNUNG VON BARBITURAT-GEMISCHEN

Gemisch Nr.	Substanzen Nr. aus Tabelle I	Fliessmittel	Trennung
1	16–12–10	I	vollständig
2	2–14	III	vollständig
3	12–5–14	II	vollständig
4	4–3	II	vollständig
5	12–7–11	I	vollständig
6	12–4–13	I	vollständig
7	4–1–8	II	1–8 unvollständig
8	12–6–3	I	3–6 unvollständig
9	12–1–3–8	I	1–3 unvollständig
10	12–1	I	vollständig
11	12–15	I	vollständig
12	12–17	II	vollständig
13	18–12	I	vollständig
14	6–9	I	vollständig

aufteilen. Einzig bei den Gemischen 7, 8 und 9 überlappten sich einzelne Zonen teilweise. Die übrigen Trennungen verliefen vollständig.

Laboratorium der Interkantonalen Kontrollstelle für Heilmittel, Bern (Schweiz) M. SAHLI
M. OESCH

[1] M. SAHLI UND M. HUBER, *Sci. Pharm.*, 27 (1959) 271.
[2] E. STAHL, *Pharmazie*, 11 (1956) 633; *Chemiker-Ztg.*, 82 (1958) 323; *Pharm. Rundschau*, 2 (1959) 1.
[3] G. MACHATA, *Mikrochim. Acta*, (1960) 79.
[4] J. BÄUMLER UND S. RIPPSTEIN, *Pharm. Acta Helv.*, 36 (1961) 382.
[5] M. FRAHM, A. GOTTESLEBEN UND K. SOEHRING, *Arzneimittel-Forsch.*, 11 (1961) 1008.
[6] H. EBERHARDT, K. J. FREUNDT UND J. W. LANGBEIN, *Arzneimittel-Forsch.*, 12 (1962) 1087.
[8] G. MACHATA UND W. KISSER, *Arch. Toxikol.*, 19 (1962) 327.
[7] J. REISCH, H. BORNFLETH UND J. RHEINBAY, *Pharm. Ztg., Ver. Apotheker-Ztg.*, (1963) 1182.
[9] H. P. KLÖCKING, *Arch. Toxikol.*, 19 (1961) 79.

Eingegangen den 21. November 1963

A method for the isolation of mono- and di-hydric alcohols from complex mixtures

In natural and synthetic mixtures, long chain saturated mono- and di-hydric alcohols are usually found associated with other classes of compounds of comparable polarity. Consequently, methods for the isolation of gram-amounts of these substances by column chromatography are time consuming and often require the use of elaborate equipment. In contrast, fatty alcohols are more conveniently separated from complex mixtures as their nitrate derivatives.

Nitrate derivatives of saturated alcohols may be rapidly prepared at room temperature by reaction of the hydroxyl group with acetyl nitrate[1]. These derivatives are quantitatively denitrated to the parent hydroxy compounds by hydrogenation or by reduction with lithium aluminum hydride[2].

The nitrates, which are slightly more polar than the corresponding hydrocarbons, have little affinity for polar adsorbents (*e.g.*, silicic acid). They are, therefore, more readily separable from complex mixtures than are the alcohols. Acetyl nitrate also reacts with olefinic compounds to form relatively polar products that do not interfere with the chromatography of fatty nitrates[3]. Consequently, saturated alcohols are readily fractionated as their nitrates from all olefinic compounds.

In the present work, a simple and rapid method is reported for the isolation of gram-amounts of saturated mono- and di-hydric alcohols by chromatography of their nitrate derivatives. Mixtures containing the alcohols are dissolved in a solution of acetyl nitrate in acetic anhydride–acetic acid. The crude products of the reaction are then placed on a column of silicic acid, and the nitrates are eluted with petroleum hydrocarbons. After removal of the solvent, the nitrates are converted to the parent hydroxy compounds.

Specific example

A fraction of alcohols (*n*-octadecyl alcohol, 1,2-octadecanediol, and 1,10-decanediol) was isolated from a model mixture (Table I) that contained a variety of other polar compounds as well as 11-tricosene. The procedure was as follows: 21 ml (0.34 mole) of colorless nitric acid was added slowly to 294 ml of acetic anhydride. The temperature

TABLE I

MODEL MIXTURE CONTAINING MONO- AND DI-HYDRIC ALCOHOLS

Compound	*% Composition*
n-Octadecyl alcohol	33.71
1,2-Octadecanediol	7.08
1,10-Decanediol	13.49
Monostearin	8.43
Dipalmitin	2.90
DL-9,10-Dihydroxystearic acid	7.42
Methyl 12-hydroxystearate	7.76
2-Hydroxystearic acid	3.03
11-Tricosene	3.37
Methyl palmitate	9.44
Azelaic acid	3.37

was maintained at 25° by use of an ice bath and by controlling the rate of addition of the nitric acid. The model mixture (15.0 g), dissolved in glacial acetic acid, was added over a period of 20 min to the acetyl nitrate solution. The reaction was allowed to continue for 30 min at 25°. It was then stopped by pouring the reaction mixture over 300 g of crushed ice. After hydrolysis was complete, the products were extracted with diethyl ether and the resulting solution was washed until neutral. The solution was then dried over anhydrous sodium sulfate and the crude product was obtained by evaporation of the solvent.

The crude product was then dissolved in 35 ml of petroleum hydrocarbons and the resulting solution was placed on a column that contained a 3:1 ratio of silicic acid (Mallinckrodt, chromatographic grade) to crude mixture. The nitrates (10.0 g) were then recovered by elution with 350 ml of petroleum hydrocarbons. Thin-layer chromatography (TLC)[4] and infrared spectrometry[5] indicated that the fraction was composed only of nitrate derivatives of the original alcohols (Fig. 1).

This fraction was further resolved into its components by column chromatography using a ratio of 10:1 silicic acid to nitrates. The *n*-octadecyl nitrate was obtained by elution with 160 ml of petroleum hydrocarbons. The two dinitrate derivatives were isolated together in one fraction by elution with a slightly more polar solvent system: petroleum hydrocarbons (30–60°)–diethyl ether (90:10). Complete separation of the 1,10-dinitrato-decane from the 1,2-dinitrato-octadecane was obtained by preparative TLC[4].

The nitrate derivatives were dissolved in ethyl acetate and hydrogenated for 2 h at 2 atm over a platinum oxide catalyst. TLC of the hydrogenated products indicated complete denitration to the parent alcohols.

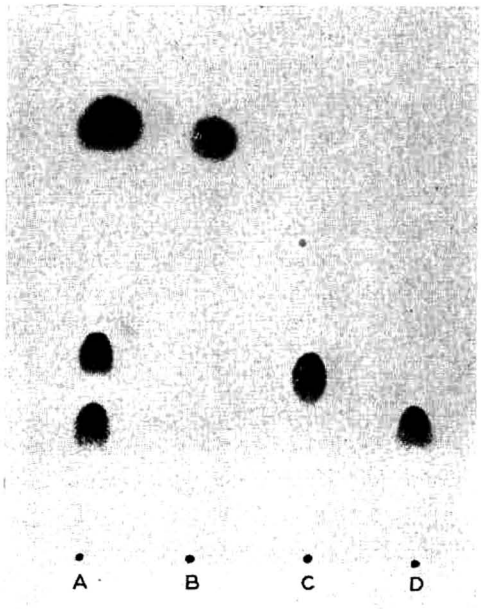


Fig. 1. Thin-layer chromatogram of fraction of nitrate derivatives of mono- and di-hydric alcohols obtained by column chromatography. (A) Fraction of nitrates; (B) *n*-octadecyl nitrate; (C) 1,2-dinitrato-octadecane; (D) 1,10-dinitrato-decane. Adsorbent: Silica Gel G. Eluent: petroleum hydrocarbons (30–60°). Indicator: 50% sulfuric acid, charring.

Discussion

The method described has been successfully applied to the fractionation of gram-amounts of mono- and di-hydric alcohols from complex mixtures. In addition, it should be adaptable to the purification of polyhydroxy alcohols containing more than two hydroxyl groups on the hydrocarbon chain. Isomeric diols have been nitrated and denitrated without change in configuration[2]. The present method, therefore, could be applied to the isolation of configurational isomers from natural and synthetic mixtures.

Bureau of Commercial Fisheries Technological Laboratory,
U.S. Fish and Wildlife Service,
Seattle, Wash. (U.S.A.)

JOHN C. WEKELL
CLIFFORD R. HOULE
DONALD C. MALINS

[1] D. C. MALINS, J. C. WEKELL AND C. R. HOULE, *J. Am. Oil Chemists' Soc.*, 41 (1964) 44.
[2] L. M. SOFFER, E. W. PARROTTA AND J. DIDOMENICO, *J. Am. Chem. Soc.*, 74 (1952) 5301.
[3] D. C. MALINS AND C. R. HOULE, *J. Am. Oil Chemists' Soc.*, 40 (1963) 43.
[4] H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.
[5] D. C. MALINS, J. C. WEKELL AND C. R. HOULE, *Anal. Chem.*, 36 (1964) 658.

Received December 9th, 1963

Celite–starch for thin-layer chromatography

Silica gel G[1], silicic acid[2], cellulose powder[3], kieselguhr G[4] and aluminium oxide[5] have been used on chromatoplates for the separation of organic and inorganic compounds. Chromatoplates with these coatings have been effective in the separation of many hydrophobic compounds but only a few reports describe their use with hydrophylic substances. This report describes a low cost coating for chromatoplates which is particularly useful in the separations of hydrophylic compounds.

The coating is a mixture of Celite 535 and starch gelatinized in sodium hydroxide. After the coating is dry on the plate the alkali present is neutralized, in some instances, by spraying the coated and dried plate with acetic acid. In both cases the adhesion of the coating to the plate is good. Reducing sugars, methyl glycosides, amino acids, purines, pyrimidines and nucleosides are separated by using 90% aqueous methyl ethyl ketone (S_2) or 90% aqueous isopropanol (S_1) as irrigants.To detect the components on the chromatoplates the usual spray reagents for paper chromatography may be used.

TABLE I

R_F VALUES OF COMPOUNDS ON CELITE–STARCH CHROMATOPLATES

Solvents: S_1 = Isopropanol–water (90:10 v/v); S_2 = Methyl ethyl ketone–water (90:10 v/v); the time needed for S_1 to run a distance of 12 cm was 25 min, and for S_2 10 min. Detection: ASN = ammoniacal silver nitrate solution[6]; AT = saturated solution of antimony trichloride in chloroform[7]; NIN = 0.3% solution of ninhydrin in butanol[8]; U.V. = ultraviolet.

Compound	*Amount applied (μg)*	R_F		*Detection*	*Color*
		S_1	S_2		
D-Glucose	5	0.28		ASN	Black
Maltose	5	0.12		ASN	Black
L-Rhamnose	5	0.92		ASN	Black
D-Ribose	5	0.50		ASN	Black
D-Fructose	5	0.31		ASN	Black
Methyl α-D-glucoside	15		0.50	AT	Brown
Methyl α-D-galactoside	15		0.54	AT	Brown
Methyl α-L-rhamnoside	15		0.88	AT	Brown
Methyl α-D-xyloside	15		0.78	AT	Brown
Methyl α-D-mannoside	15		0.46	AT	Brown
L-Glycine	2	0.30		NIN	Purple
D-Alanine	2	0.51		NIN	Purple
L-Cystein	3	0.15		NIN	Purple
L-Valine	2	0.77		NIN	Purple
DL-Lysine	2	0.28		NIN	Purple
DL-Serine	3	0.32		NIN	Purple
L-Phenylalanine	4	0.85		NIN	Purple
Adenine sulfate	5	0.96		U.V.	
Dihydrouracil	5	0.74		U.V.	
Cytidine	5	0.55		U.V.	
2-Amino-4-hydroxy-pyrimidine	5	0.94		U.V.	
5-Methylcytosine HCl	5	0.88		U.V.	
Adenosine	5	0.77		U.V.	
Uridine	5	0.39		U.V.	
Inosine	5	0.21		U.V.	
Adenosine 5-phosphoric acid	5	0.15		U.V.	
Thymidine	3		0.85	AT	Blue
5-Methyl-deoxycytidine	3		0.64	AT	Blue
Deoxycytidine HCl	3		0.50	AT	Blue
Thymidine 5-monophosphoric acid	3		0.00	AT	Blue

For the preparation of the chromatoplates a suspension of 18 g of 200-mesh Celite 535 (Johns-Mansville Company) in 100 ml of 0.25 *N* sodium hydroxide solution was mixed with a suspension of 1.5 g of powdered potato starch in 20 ml of water and homogenized by mixing for 1–2 min. The mixture was immediately coated on clean and dry 20 cm × 20 cm glass plates with an applicator model S-11 (Brinkmann Instruments) which was adjusted to give a layer of 500 μ thickness. The plates were dried at 25° for 16 h. The chromatoplates were irrigated at 25° in chambers saturated with vapors of the solvents. Samples were applied 2 cm from the edge of the glass plate. The ascending irrigant technique was used and the best results were obtained when the plates were allowed to rest on a 5 mm thick layer of cotton soaked with the irrigant. Some results are shown in Table I.

Deoxy-D-ribose nucleosides were detected by spraying the plates with a saturated solution of antimony trichloride. A fairly stable deep blue color was produced upon heating the plates at 100° for 10 min. Under the same conditions D-ribose nucleosides produce a yellow brown color.

Since the optimum pH for the detection of amino acids with ninhydrin is about neutral[9], the chromatoplates for the separation of these acids were sprayed with 20 % solution of acetic acid and dried at 25° before use. Purines were not detectable on the chromatoplates by direct observation in U.V. light and another technique was employed for their observation. Chromatoplates containing these substances were dried and sprayed with water. Then a water wetted piece of Whatman No. 1 filter paper was applied to the chromatoplates and covered with glass plate. The plates were heated gently on steam bath for 2–3 min. The plates were separated and the paper pulled off and dried. Purines and pyrimidines were transferred to the paper and detected by a U.V. light (SL Mineralight, Model 2537 with a 253.7 mμ short wave filter).

Department of Biochemistry, Purdue University, Lafayette, Ind. (U.S.A.)

BARUCH SHASHA
ROY L. WHISTLER

[1] E. STAHL, *Arch. Pharm.*, 293 (1960) 531.
[2] R. H. REITSEMA, *Anal. Chem.*, 26 (1954) 960.
[3] E. V. DYATLOVITSKAYA AND L. D. BERGEL'SON, *Dokl. Akad. Nauk SSSR*, 145 (1962) 325.
[4] E. STAHL AND U. KALTENBACH, *J. Chromatog.*, 5 (1961) 351.
[5] E. MUTSCHLER AND H. ROCHEIMEYER, *Arch. Pharm.*, 292 (1959) 610.
[6] M. S. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
[7] E. STAHL, *Chemiker-Ztg.*, 82 (1958) 323.
[8] H. K. BERRY AND L. CAIN, *Arch. Biochem.*, 24 (1949) 179.
[9] S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.

Received October 11th, 1963

Inorganic ion exchangers for thin-layer chromatography

The purpose of this communication is to report some preliminary results obtained with several inorganic ion exchangers in thin-layer chromatography. Although TLC is now a well-established analytical method, there have been relatively few papers dealing with the separation of inorganic ions[1,2]. The properties of inorganic ion exchangers have been reviewed by KRAUS *et al.*[3] and AMPHLETT[4]. Recently, a paper relating the values of distribution constants *versus* pH for 60 metal ions has been published[5]. The unusual selectivities of these materials for various ions led us to believe that a variety of useful separations could be made.

Preparation and stability of plates

The materials chosen for study were zirconium phosphate (ZP) and hydrous zirconium oxide (HZO). Both are cation exchangers, but the latter exhibits anion exchange properties in acid media. Analytical grade ion exchange crystals—Bio-Rad ZP-1 (hydrogen form) and Bio-Rad HZO-1 (ammonium form) for TLC*—were used in these experiments. The material can be used without binder if the precaution is taken to place a layer of glass wool on the bottom of the developing jar to prevent sloughing off of the material. In this study, 3% corn starch was used as a binder. A slurry was prepared by mixing 20 ml of water with 20 g of the exchanger. This mixture was heated in a double boiler until a thick gel formed. A small amount of water was then added to form a slurry suitable for spreading on the plates. A plate thickness of 500 μ was used. The plates were dried at 40° for 30 min or overnight at room temperature. Both ZP and HZO formed extremely hard plates.

Separations on HZO

For most separations, the ammonium or cation form of the exchanger was found to be superior to the HCl (anion) form. The latter can readily be prepared by washing on a Buchner funnel with 1 *M* HCl followed by water to remove excess acid. Approximately 0.001 ml of 0.1 *M* solutions of Ni, Co(II), Pb, Fe(III), Ag, Hg, Cd and Cu were applied to one end of the TLC plate. Table I shows the R_F values obtained by elution of the ammonium form of HZO with several solvents.

TABLE I

R_F VALUES OF METAL IONS ON THE AMMONIUM FORM OF HZO
(Development time: 10 cm in 10 min)

	Ni	*Co*	*Pb*	*Fe*	*Ag*	*Hg*	*Cd*	*Cu*
0.01 *M* HCl	1.0	1.0	0–0.1	0	0	0.1	0.75	0
0.1 *M* NH_4NO_3	0.9	0.9	0	0	0	0.15	0.6	0
2.0 *M* NH_4NO_3	0.5	0.5	0	0	0	0.9	0.3	0

The spots were detected by spraying with an ammoniacal solution of ammonium sulfide. It is interesting to note that a variation in HCl concentration from 0.001 to

* Bio-Rad Laboratories, 32nd & Griffin Avenue, Richmond, Calif.

TABLE II

R_F VALUES OF METAL IONS ON THE HCl FORM OF HZO
(Development time: 10 cm in 45 min)

	Ni	*Co*	*Pb*	*Fe*	*Ag*	*Hg*	*Cd*	*Cu*
0.01 *M* HCl	0.9	0.9	0.5	0	0	0.1	0.9	0.9
0.1 *M* NH_4Cl	0.95	0.95	0.4	0	0	0.1	0.8	0.95

0.1 *M* results in little change in the R_F values. However, Table I indicates that a change in the NH_4NO_3 concentration does result in some changes in the R_F values.

Table II shows some of the results obtained with the HCl form of HZO. In these cases, mercury was the only ion which showed any variation of R_F values with changes in the eluent concentration and that only with NH_4Cl.

Separations on ZP

Table III lists the R_F values obtained on eluting the hydrogen form of ZP with 0.1 *M* HCl.

TABLE III

R_F VALUES OF METAL IONS ON THE HYDROGEN FORM OF ZP
(Development time: 10 cm in 50 min)

	Ni	*Co*	*Pb*	*Fe*	*Ag*	*Hg*	*Cd*	*Cu*
0.1 *M* HCl	0.65–0.95	0.3–0.6	0	0–0.1	0	0.85	0.4	0.1

It appears that many of the metal ions have a tendency to tail with the eluents used. However, with the exception of nickel and cobalt, the tailing is not serious. A very specific separation was obtained for mercury from the rest of the ions. This was performed on the ammonium form of ZP, obtained by neutralizing the slurry with NH_4OH before forming the plate, and eluting with 3 *M* NH_4Cl–NH_4OH solution.

In conclusion, inorganic ion exchangers can be adapted to the technique of thin-layer chromatography and several very specific separations have been demonstrated. Furthermore, it is expected that these materials should be especially applicable to the separation of alkali and alkaline earth ions owing to their vast differences in selectivities towards these ions.

Bio-Rad Laboratories,
Richmond, Calif. (U.S.A.)

BURTON A. ZABIN
CHARLES B. ROLLINS

[1] H. SEILER, *Helv. Chim. Acta*, 45 (1962) 381.
[2] H. SEILER AND M. SEILER, *Helv. Chim. Acta*, 43 (1960) 1939.
[3] K. A. KRAUS, H. O. PHILLIPS, T. A. CARLSON AND J. S. JOHNSON, *2nd Intern. Conf. Peaceful Uses At. Energy, Geneva, 1958*, Vol. 28, Paper 1832, p. 3, United Nations, New York, 1958.
[4] C. B. AMPHLETT, *2nd Intern. Conf. Peaceful Uses At. Energy, Geneva, 1958*, Vol. 28, Paper 271, p. 17, United Nations, New York, 1958.
[5] W. J. MAECK, M. E. KUSSY AND J. E. REIN, *Anal. Chem.*, 35 (1963) 2087.

Received December 16th, 1963

Chromatographic separation of morphine, normorphine and nalorphine

A simple method for the chromatographic separation of morphine, normorphine and nalorphine is important for the metabolic study of these alkaloids. BROSSIE, HÄFLIGER AND SCHNIDER[1] developed a chromatographic method which gives a good separation of these substances and has been extensively employed by several research workers. This method is unfortunately rather time-consuming and tedious, it requires buffered paper and highly purified solvents and it gives compact spots, in a rather small range of alkaloid concentrations.

We have developed two new chromatographic methods, one on paper and another on thin layers of silica gel.

The paper chromatographic method utilizes Whatman No. 1 paper without previous treatment. The solvent mixture (solvent 1) is pyridine–ethyl acetate–water (23:75:16.5). Resolution is accomplished in about 6 h. The R_F values at 20° for morphine, normorphine and nalorphine are given in column 1 of Table I.

TABLE I

Alkaloid	R_F	
	Paper Solvent 1	*Thin layer Solvent 2*
	(1)	(2)
Morphine	0.80	0.05
Normorphine	0.35	0.42
Nalorphine	0.91	0.60

The thin-layer method is performed on neutral Silica gel G prepared according to STAHL (E. Merck AG, Darmstadt). The solvent mixture (solvent 2) is chloroform–isopropyl alcohol (1:3). Resolution is obtained in about 90 min. The R_F values at 20° are shown in column 2 of Table I.

Under shortwave (2537 Å) ultraviolet light, morphine and normorphine spots show blue fluorescence while nalorphine shows absorption only. When the chromatograms are sprayed with potassium iodoplatinate solution[2] morphine gives a blue, normorphine a violet blue, and nalorphine a blue coloration. On silica gel the blue spot of nalorphine disappears in about an hour, leaving a white spot.

Instituto de Farmacología, Universidad de Chile, Santiago (Chile) ANA PENNA-HERREROS

[1] A. BROSSI, O. HÄFLIGER AND O. SCHNIDER, *Arzneimittel-Forsch.*, 5 (1955) 62.
[2] G. MANNERING, A. DIXON, N. CARROL AND O. COPE, *J. Lab. Clin. Med.*, 44 (1954) 292.

Received September 2nd, 1963

Paper chromatography of the denaturation and first degradation products of collagen

The main products of primary degradation and denaturation of tropocollagen have been recently separated by means of ultracentrifugation[1] and also by ion-exchange column chromatography on carboxymethyl-cellulose[2]. The results of these separation techniques were amplified by the work of GRASSMANN, HANNIG AND ENGEL dealing with the primary structure of collagen[3]. These authors, while describing some important sequences in the collagenous structure, have shown the relation between the α, β and γ products of denaturation. These fractions were described for the first time by PIEZ *et al.*[2,4], who in a more detailed study[4] have established that the α fraction is not a uniform fraction, but that there are two α fractions, called α_1 and α_2. By combining these two fractions one can obtain the so-called β_1 and β_2 fractions.

In 1962 SCHLEYER[5] gave some evidence for the existence of smaller subunits of the α fractions, and described them as the A and B fractions. BLUMENFELD AND

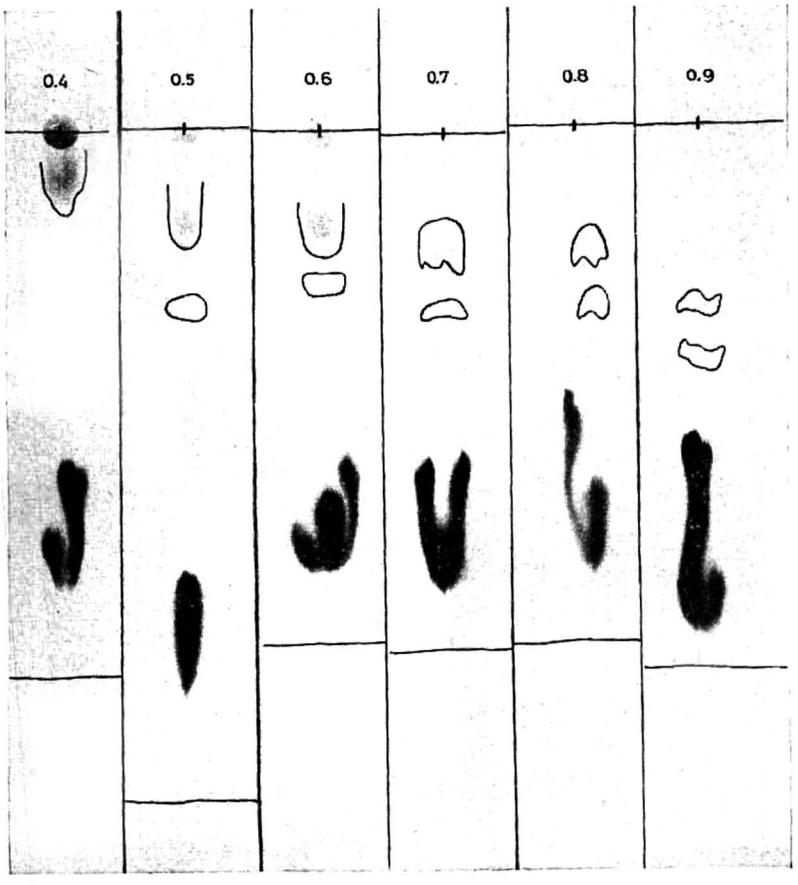


Fig. 1. Separation of denaturation and primary degradation products of collagen. From the front to the start $\alpha_1 + \beta_2$, β_1, α_2. Solvent: isoamyl alcohol–acetic acid–water 1 : 2 : x. The number designates water content of the solvent mixture.

GALLOP[6] split the α fractions into four subunits each with a molecular weight of about 25,000. These subunits are bound together by means of ester linkages.

As both ultracentrifugation and column chromatography are time-consuming operations when one examines larger series of samples, we tried to develop a paper chromatographic technique for the separation of these collagenous subunits. In a previous paper[7] a simple centrifugal chromatographic technique for the separation of α and β fractions of collagen was described. In the present paper we tried to improve the quality of separation and to separate distinctly the fractions with lower R_F, *e.g.*, β_1 and α_2 and the so-called B fraction from the α_1 and A fractions.

Experimental and results

In our experiments acid-soluble calf-skin collagen was used. Solutions of collagen were prepared by the technique of unlimited swelling that has been described elsewhere[8]. For the chromatographic separation the collagenous solution was denatured by heating at 37–50° to obtain a mixture of the α and β fractions. A solution containing

TABLE I

R_F VALUES OF COLLAGEN FRACTIONS IN VARIOUS SOLVENT SYSTEMS (DEPENDENCE ON THE WATER CONTENT IN THE SOLVENT)

(a) Isopropyl alcohol–acetic acid–water 1 : 2 : x

Fraction	R_F			
	$x = 0.6$	$x = 0.7$	$x = 0.8$	$x = 0.9$
$\alpha_1 + \beta_2 + A$	1.0	1.0	1.0	1.0
$B + \beta_1$	0.32	0.39		
α_2	0.24	0.28		

(b) n-Butyl alcohol–acetic acid–water 1 : 2 : x

	$x = 0.6$	$x = 0.7$	$x = 0.8$	$x = 0.9$
$\alpha_1 + \beta_2 + A$	0.84	0.87	0.86	1.0
$B + \beta_1$	0.09	0.33	0.35	0.44
α_2		0.22	0.24	

(c) Isobutyl alcohol–acetic acid–water 1 : 2 : x

	$x = 0.6$	$x = 0.7$	$x = 0.8$	$x = 0.9$
$\alpha_1 + \beta_2 + A$	0.84	0.75	0.77	0.81
$B + \beta_1$	0.21	0.27	0.35	
α_2		0.18	0.26	

(d) Isoamyl alcohol–acetic acid–water 1 : 2 : x

	$x = 0.4$	$x = 0.5$	$x = 0.6$	$x = 0.7$	$x = 0.8$	$x = 0.9$
$\alpha_1 + \beta_2 + A$	0.74	0.75	0.81	0.81	0.79	0.85
$B + \beta_1$	0.10	0.27	0.30	0.33	0.34	0.43
α_2		0.12	0.21	0.22	0.24	0.34

A and B fractions was prepared according to SCHLEYER[5]. 0.5 μl samples of 1 % protein solution were spotted on Whatman paper No. 1. A series of solvents of the alcohol–acetic acid–water type were used as the mobile phases, the lower aliphatic alcohols up to C_5 being used as the alcoholic component. The ratio alcohol–acetic acid was 1:2 and the content of water varied between 0.5–1 (parts by volume). Detection was performed by means of bromophenol blue (0.5 % in methanol, saturated with $HgCl_2$) and the excess of the dye was washed out with 2 % acetic acid in water. In Fig. 1 the possibilities of this chromatographic process are shown. A survey of R_F values is given in Table I.

It can be concluded that a good separation either of the α_1, α_2 and β_1 fractions or a distinct separation of A and B fractions is obtained by means of paper chromatography. However, the separation of mixtures containing both types of degradation products (α and β fractions together with A and B fractions) is impossible. Also a separation of the α_1 fraction from the β_2 fraction was not achieved.

Central Research Institute of Food Industry, Prague-Smíchov (Czechoslovakia)

ZDENĚK DEYL
JAN ROSMUS

[1] V. N. ORECHOVITCH AND V. O. SPHIKITER, *Dokl. Akad. Nauk SSSR*, 101 (1955) 529.
[2] K. A. PIEZ, E. WEIS AND M. S. LEWIS, *J. Biol. Chem.*, 235 (1960) 1987.
[3] W. GRASSMANN, K. HANNIG AND J. ENGEL, *Z. Physiol. Chem.*, 324 (1961) 284.
[4] K. A. PIEZ, M. S. LEWIS, G. R. MARTIN AND J. GROSS, *Biochim. Biophys. Acta*, 53 (1961) 596.
[5] M. SCHLEYER, *Z. Physiol. Chem.*, 329 (1962) 97.
[6] O. O. BLUMENFELD AND P. M. GALLOP, *Biochemistry*, 1 (1962) 947.
[7] Z. DEYL AND J. ROSMUS, *J. Chromatog.*, 11 (1963) 110.
[8] Z. DEYL, J. ROSMUS AND O. VANČÍKOVÁ, *Czechoslovak. Pat.* 2049/63.

Received November 22nd, 1963

J. Chromatog., 14 (1964) 537–539

Paper chromatography of polyphenyls
Quantitative determination of diphenyl in the presence of polyphenyls

Quantitative methods of determination for diphenyl are based on either fractional distillation or the water solubility of diphenyl. The latter technique, as described by SILVERMAN AND SHIDELER[1], has some disadvantages, when polyphenyls (terphenyl and higher polyphenyls) are also present.

In an earlier paper[2] the present authors described a method for the chromatographic separation of diphenyl and isomer terphenyls, depending on the different speeds of sulphonation of polyphenyls and the different migration speeds of sulphonic acids on non-impregnated paper.

By choice of suitable experimental conditions it is possible to use this method for the quantitative determination of diphenyl in polyphenyl mixtures.

Experimental

Sulphonation of polyphenyls. 0.25 g of polyphenyl mixture are mixed with 1.5 ml of concentrated sulphuric acid (d = 1.84, Merck analytical reagent grade) and heated in a thermostat at 150° for 7 h.

The sulphonic acid mixture is diluted with a little distilled water, transferred to a small volumetric flask (25 ml) and made up to volume. The yield of the disulphonic acids of the various polyphenyls so obtained is quantitative.

The same procedure can be used for the preparation of the pure 4,4'-diphenyldisulphonic acid. Fig. 1 shows the U.V. absorption spectrum of the 4,4'-diphenyldisulphonic acid (Beckman DB spectrophotometer). The calibration curve is prepared by measuring the absorption of accurately weighed portions of 4,4'-diphenyl-disulphonic acid, diluted as required, at 265 mμ, slit width 0.5 mm, against a blank of about 6 % aqueous solution of H_2SO_4.

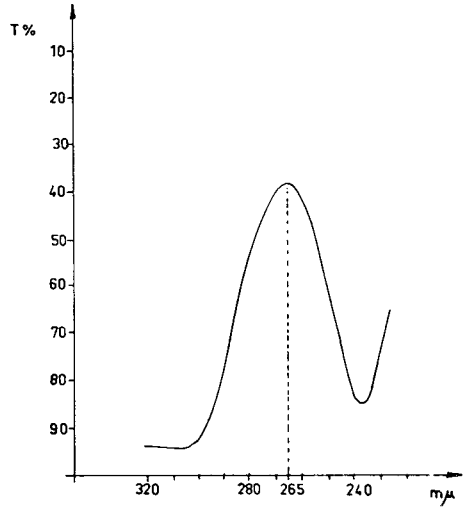


Fig. 1. U.V. absorption spectrum of 4,4'-diphenyldisulphonic acid (aqueous solution $2.5 \cdot 10^{-3}$ g/l); Beckman DB spectrophotometer.

Chromatographic technique. The chromatographic separations are carried out by the ascending technique, on 6 × 53 cm strips of Whatman chromatographic paper (No. 20).

The developing solution, *n*-butanol–methanol–3 *N* aqueous $(NH_4)_2CO_3$ solution (4:3:2), is put in a glass chromatographic chamber (31 × 21 × 38 cm) 20 h before the introduction of the strips, thus allowing ample time for equilibrium saturation of the gas phase to be reached.

Various quantities (0.008–0.1 ml), taken as required by the different concentrations of diphenyl in the sulphonic acid solution, are placed on the paper by a micrometric volume measuring device (accuracy $\pm$ $1.8 \cdot 10^{-4}$ ml, Desaga GmbH, Heidelberg).

Two identical spots are deposited on every strip and the strips are developed by the above-mentioned solvent, for 40 h at 20°. The strips are dried in an air oven at 70° for 30 min, and are cut lengthwise into two. One of the two halves is sprayed with a 0.1 % alcoholic solution of methyl red, diluted 1:5 with phosphate buffer (pH = 7), in order to locate the two spots (4,4'-diphenyldisulphonic acid, $R_F = 0.37$ and polyphenyl disulphonic acids, $R_F = 0.23$).

The region around where the 4,4'-diphenyldisuphonic acid is located is cut out from the other half of the strip and this piece of paper is further cut in smaller pieces and extracted (by distilled water), in a special extraction apparatus with reflux condenser, for 3 h. The same quantity of paper is extracted under the same conditions for a blank for the spectrophotometric analysis. The extraction liquids and wash waters are made up to 25 ml in volumetric flasks. The diphenyl is determined by its absorption at 265 mμ.

Results and discussion

The sulphonation process of heating the mixture of diphenyl and isomeric terphenyls with concentrated H_2SO_4 at 150° for 7 h leads to quantitative sulphonation of all the components to disulphonic acids. The R_F value of the 4,4'-diphenyldisulphonic acid, under the above-mentioned conditions, is 0.37, whereas that for the terphenyldisulphonic acids is 0.23.

Other polyphenyls do not interfere, the R_F of their sulphonic acids being less than 0.23 and their spot is often masked by the excess H_2SO_4, in a large, diffuse spot at the start.

TABLE I

COMPOSITION OF SYNTHETIC MIXTURES MADE FOR TESTING THE METHOD

Mixtures	*Diphenyl* mg	*o-Terphenyl* mg	*m-Terphenyl* mg	*p-Terphenyl* mg	*Diphenyl* %
1	60.54	79.09	59.23	47.69	24.55
2	48.39	64.25	64.57	67.53	19.77
3	25.12	129.41	65.74	22.95	7.56
4	13.67	66.82	187.44	0	5.10
5	5.13	49.18	127.58	65.04	2.07
6	3.28	111.47	63.80	64.89	1.34

Table I shows the composition of synthetic terphenyl mixtures, with the various diphenyl to terphenyl ratios. Table II shows the results of a number of quantitative determinations made by the method described here. Every result is the average of ten independent determinations.

Volumes between 0.008–0.12 ml of solution, depending on the various diphenyl

TABLE II

ANALYTICAL RESULTS FOR DIPHENYL IN A POLYPHENYL MIXTURE

(Average of 10 independent determinations for each diphenyl concentration)

No.	*Diphenyl present* %	*Diphenyl found* %	*Standard deviation*
1	24.55	24.52	± 1.32
2	19.77	20.85	± 1.01
3	7.56	7.50	± 0.45
4	5.10	5.14	± 0.32
5	2.07	2.06	± 0.16
6	1.34	1.30	± 0.08

concentrations, are used to obtain a clearly visible spot (10 γ) and to give enough material for spectrophotometric determination.

Some care is necessary when depositing the drop of solution, in order to prevent the H_2SO_4 from making a hole in the paper during the drying of the spots.

Acknowledgements

Thanks are due to the directors of SORIN for permission to publish this paper. The authors wish to thank Dr. E. ZIMMER who read the manuscript and gave helpful criticism.

SORIN, Centro Ricerche Nucleari,
Saluggia (Italy)

E. DENTI
M. P. LUBOZ
A. MASSAGLIA

[1] L. SILVERMAN AND M. E. SHIDELER, *Anal. Chim. Acta*, 18 (1958) 540.
[2] E. DENTI, M. P. LUBOZ AND A. MASSAGLIA, *J. Chromatog.*, 11 (1963) 339.

Received December 2nd, 1963

J. Chromatog, 14 (1964) 539–542

Chromatographic separation of nucleotides and nucleosides

I would like to report three chromatographic systems that have been found useful in separating various pyrimidine nucleotides and nucleosides. Of the two systems using DEAE-cellulose paper, one employs borate complexing[1] to separate ribonucleotides from nucleotides lacking *cis*-hydroxyl groups and the other takes advantage of the difference in mobility in acid between cytosine- and uracil-containing compounds[2]. The third system is a modification of the ammonium isobutyrate system[3] introduced by Pabst Laboratories for better resolution of GMP*, GDP and GTP[4].

System 1

The developer used is 0.2 *M* ammonium bicarbonate–0.005 *M* sodium tetraborate. The paper is obtained from Whatman (designated DE-20), a DEAE-cellulose with a nominal capacity of 0.4 mequiv./g. At 24° the solvent front moves 40 cm in 6 h.

System 2

The developer used is 0.05 *M* formic acid. The DE-20 paper is converted to the formate form by irrigating with 1 *M* formic acid followed by water. The papers are dried and stored at room temperature. The solvent front moves 40 cm in 1.5 h at 24°.

* Abbreviations: GMP = guanosine monophosphate; GDP = guanosine diphosphate; GTP = guanosine triphosphate; CMP = cytidine monophosphate; deCMP = deoxycytidine monophosphate; CDP = cytidine diphosphate; CTP = cytidine triphosphate; UMP = uridine monophosphate; UDP = uridine diphosphate; UTP = uridine triphosphate; TMP = thymidine monophosphate; TDP = thymidine diphosphate; TTP = thymidine triphosphate.

System 3

The developer used consists of isobutyric acid, conc. NH_4OH and water, 577 ml, 38 ml and 385 ml, respectively.[4] The paper is Whatman No. 1 or 3 MM, and about 18 h is required for the front to move 40 cm at 24°.

The mobilities are shown in Table I. Omission of the borate from System 1 results in no changes except that the 2'- or 3'-ribonucleotides and deoxyribonucleotides migrate with the 5'-ribonucleotides.

TABLE I

R_F VALUES FOR PYRIMIDINE NUCLEOSIDES AND NUCLEOTIDES*

Compound	System		
	1	2	3
Cytidine	0.83	0.90	0.67
2',3'-CMP	0.48	0.58	0.53
5'-CMP	0.36	0.60	0.50
CDP	0.26	0.02	0.36
CTP	0.21	0.00	0.24
deCMP	0.49	0.62	0.57
Uridine	0.59	0.90	0.58
2', 3'-UMP	0.40	0.04	0.44
5'-UMP	0.30	0.04	0.39
UDP	0.19	0.00	0.24
UTP	0.15	0.00	0.20
Thymidine	0.76	0.89	0.68
TMP	0.48	0.04	0.53
TDP	0.33	0.00	0.39
TTP	0.25	0.00	0.29

* Average of R_F values obtained in two chromatograms. Individual values usually agree with the averages shown within 10%.

The DEAE-cellulose systems are very useful since the solvent movement is more rapid than in the isobutyrate system, and System 1 is not affected by the presence of salt in the sample. In System 1 minus borate the separations achieved are quite similar to those in System 3.

Further experience on DEAE-cellulose thin-layer systems has been reported by RANDERATH[5]. The resolution of the ribonucleotides of RNA was reported previously[6].

*Biology Division, Oak Ridge National Laboratory**, *Oak Ridge, Tenn. (U.S.A.)*

K. BRUCE JACOBSON

1 W. E. COHN, in E. CHARGAFF AND J. N. DAVIDSON (Editors), *The Nucleic Acids*, Vol. 1, Academic Press, New York, 1955, p. 211.
2 W. E. COHN, *J. Am. Chem. Soc.*, 72 (1952) 1471.
3 N. LÖFGREN, *Acta Chem. Scand.*, 6 (1952) 1030.
4 *Circular OR-10*, Pabst Laboratories, Inc., Milwaukee, Wisc., U.S.A.
5 K. RANDERATH, *Thin-Layer Chromatography*, Academic Press, New York, 1963.
6 K. B. JACOBSON, *Science*, 138 (1962) 515.

Received November 18th, 1963

* Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

The electrophoretic separation and detection of the isomers of ethylenediamine di-(o-hydroxyphenyl-acetic acid) metal chelates*

The medical, analytical and agricultural fields have developed an increasing number of uses for the metal chelates of ethylenediamine di-(*o*-hydroxyphenyl-acetic acid),** EDDHA, since its introduction by KROLL *et al.*[1] in 1957. These widespread uses have created the need for a better understanding of the chemical and physical properties of these metal chelates.

RYSKIEWICH AND BOKA[2] as well as HILL-COTTINGHAM[3] have made paper chromatographic separations of the *meso* and DL-racemic mixture forms of ferric-EDDHA, indicating that three separate geometric isomers of EDDHA and metal chelates of EDDHA, *meso-*, *dextro-* and *levo-*, are possible. This study is concerned with the electrophoretic migration, separation and detection of the isomeric forms of EDDHA and its metal chelates.

Experimental

Buffer

(a) B-2, Veronal buffer[4]; diethyl barbituric acid, 2.76 g and sodium diethylbarbiturate, 15.40 g/l of solution, pH 8.6.

(b) Phosphate, borate and tris-(hydroxymethyl)-aminomethane buffers either hindered migration or broadened the zones and were not used in the experiments reported here.

Paper

Uniform filter paper strips, Schleicher and Schüll 2043-A mgl and filter paper wicks, Schleicher and Schüll 430[4], were used in all experiments.

Equipment

The Beckman/Spinco Model R electrophoresis cell (Durrum type) and Model RD-2 Duostat, utilizing the constant current circuit, were used throughout this study. The experiments were performed in a constant temperature laboratory at 4 ± 2°.

Methods of detection

(*a*) *FeEDDHA*. The ferric chelate of EDDHA has a red-brown color which is stable from pH 4 to 12; hence, no supplemental color reaction is required. Ferrous ion is oxidized by EDDHA to the ferric state also forming the ferric chelate.

(*b*) *Dextrose*. To develop a tan to light brown color with dextrose in the presence of the B-2 buffer, the strips may be heated at 100° for 30 min or they may be sprayed with aniline hydrogen phthalate reagent[5] and dried for 10 min at 100° to develop a medium brown color.

(*c*) *CuEDDHA*. The emerald green color of CuEDDHA fades during electrophoresis. A spray reagent of diethyl dithiocarbamate (10 mg/ml) reacts with the copper to form a light yellow-brown zone on a white background.

(*d*) *ZnEDDHA and EDDHA*. The spray reagent used for the detection of these two compounds was a solution of 0.002 *M* ferrous ammonium sulfate and 0.03 *M*

* University of Arizona Technical Paper, No. 849.

** Also known as Chel-138, Sequestrene 138, Chel DP, N,N'-Ethylene bis-[2-(*o*-hydroxyphenyl)]-glycine and EHPG.

hydroxylamine hydrochloride. Both EDDHA and the diethylbarbituric acid buffer will oxidize ferrous ion to the ferric form. In order to elimate the yellow background caused by the oxidation of ferrous ion by diethylbarbituric acid, hydroxylamine hydrochloride was added to suppress this reaction and to allow only the oxidative chelation of ferrous ion by EDDHA, producing the characteristic red-brown color of FeEDDHA on a white background.

(*e*) *CoEDDHA and all EDDHA metal chelates.* Folin-Ciocalteau's phenol reagent[6] (2.0898 *N*) was found to react with EDDHA and its metal chelates to form a deep blue color. When this reagent is used as a spray, a deep blue color is produced on a yellow background. The yellow background is eliminated by spraying the strips with 6 *N* NaOH and drying at 100° for 15 min. Folin-Ciocalteau's phenol reagent also reacts with dextrose to form a deep blue color.

Electroosmosis

The migration distance of dextrose, applied at the origin during sample application, was used to correct the migration distances of the samples for the electroosmotic flow of the buffer.

Migration analysis

All strips were densitometered on the Beckman/Spinco Model RB Analytrol using the 450 mμ filters and a B-5 cam. The midpoint of concentration for each migration zone was determined by dividing the area under the curve for each migrating species by two. The distance between the dextrose concentration midpoint and the midpoint of the individual zones was taken as the differential migration for that species. Fig. 2 shows the curve produced and the automatic integration of the curve.

Results and discussion

Successful isomeric separation of two zones was accomplished only for EDDHA, ZnEDDHA and CuEDDHA at a constant current of 15 mA. These two zones were thought, but not confirmed, to be the meso- and DL-racemic mixture forms of EDDHA and the Zn and Cu metal chelates. The separation of EDDHA isomers, Fig. 1, required 7.5 h, yielding an isomeric separation of 17.2 mm. When the constant current was increased to 50 mA for 3 h, three zones were resolved (Fig. 2) with separations of 15.9 mm and 8.6 mm. These zones are possibly the *meso-*, *dextro-* and *levo-* isomers of EDDHA.

ZnEDDHA gave the greatest isomeric separation, 70.7 mm (Fig. 3), while CuEDDHA (Fig. 4) gave the least separation 8.6 mm, during a 7-h run.

Separation of the isomers of FeEDDHA (Fig. 5) and CoEDDHA (Fig. 6) was not accomplished at a constant current of 15 mA, but it is possible that a higher constant current (greater than 50 mA) would produce the desired isomeric separations.

Since EDDHA has a high affinity for iron, K_{ma} Fe^{3+} = 30, the separation of FeEDDHA from the EDDHA isomers was performed at 15 mA for 7.5 h. This resulted in identical differential migrations for the EDDHA isomers and an expected increase for FeEDDHA due to the increase in duration of the applied current (Fig. 7).

The differential migrations for EDDHA and its metal chelates as well as the time, current, and concentration factors for each are given in Table I.

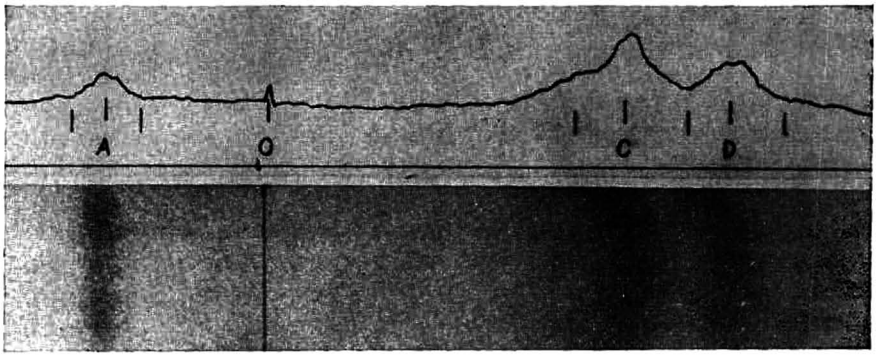


Fig. 1. Electrophoretic separation of EDDHA isomers. B-2 buffer, pH 8.6; 15 mA for 7.5 h. A = Dextrose; O = Origin; C = EDDHA′; D = EDDHA″; AO = −28.3 mm; OC = +67.6 mm; OD = +84.8 mm; AC = +95.9 mm; AD = +113.1 mm.

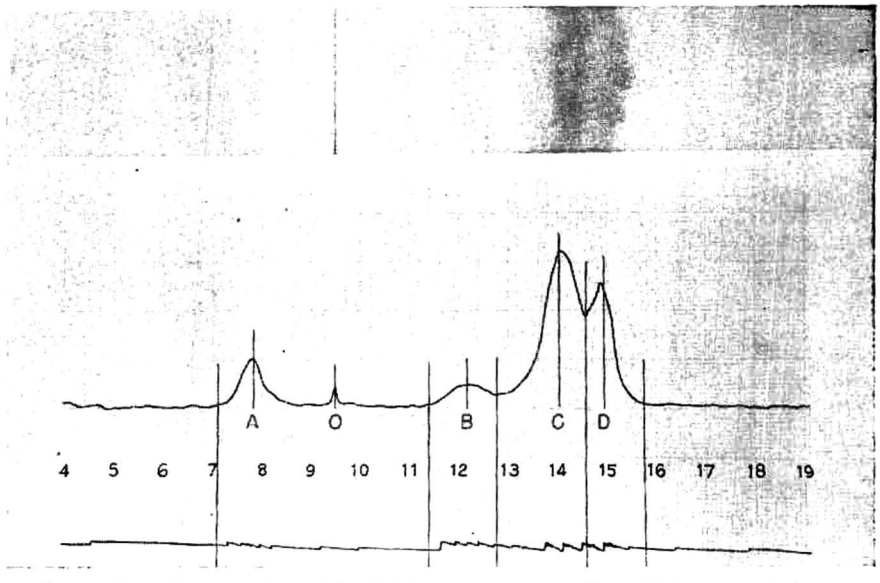


Fig. 2. Electrophoretic separation of EDDHA isomers. B-2 buffer, pH 8.6; 50 mA for 3 h. A = Dextrose; O = Origin; B = EDDHA′; C = EDDHA″; D = EDDHA‴; AO = −19.7 mm; OB = +27.4 mm; OC = +43.3 mm; OD = +51.9 mm; AB = +47.1 mm; AC = +63.0 mm; AD = +71.6 mm.

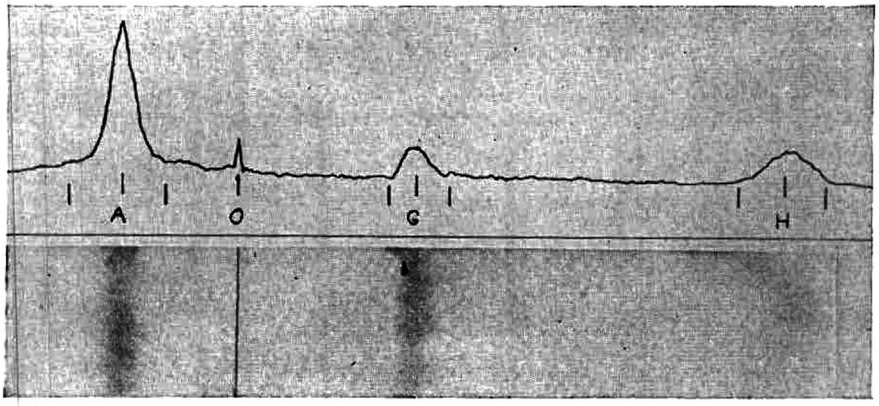


Fig. 3. Electrophoretic separation of ZnEDDHA isomers. B-2 buffer, pH 8.6; 15 mA for 7 h. A = Dextrose; O = Origin; G = ZnEDDHA′; H = ZnEDDHA″; AO = —22.8 mm; OG = +36.3 mm; OH = +107.0 mm; AG = +59.1 mm; AH = +129.8 mm.

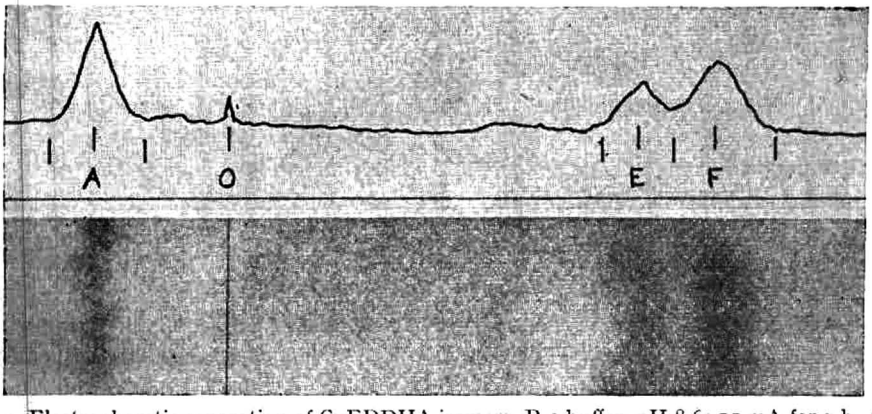


Fig. 4. Electrophoretic separation of CuEDDHA isomers. B-2 buffer, pH 8.6; 15 mA for 7 h. A = Dextrose; O = Origin; E = CuEDDHA′; F = CuEDDHA″; AO = —22.4 mm; OE = +67.5 mm; OF = +81.0 mm; AE = +89.9 mm; AF = +103.4 mm.

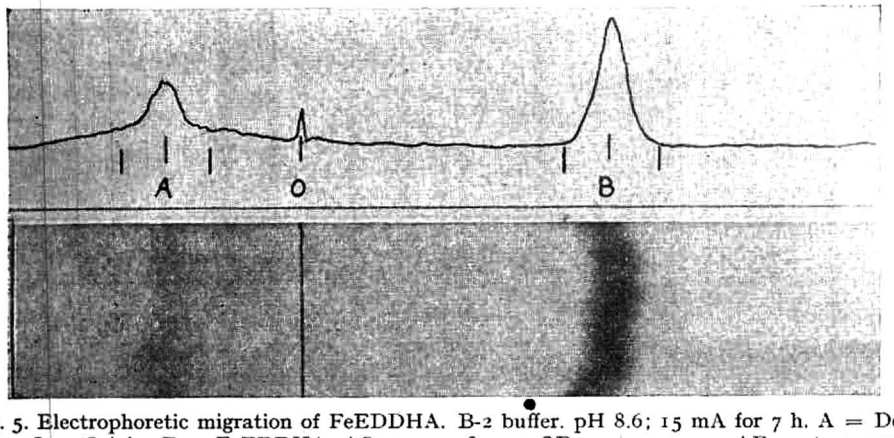


Fig. 5. Electrophoretic migration of FeEDDHA. B-2 buffer. pH 8.6; 15 mA for 7 h. A = Dextrose; O = Origin; B = FeEDDHA; AO = —23.8 mm; OB = +53.2 mm; AB = +77.0 mm.

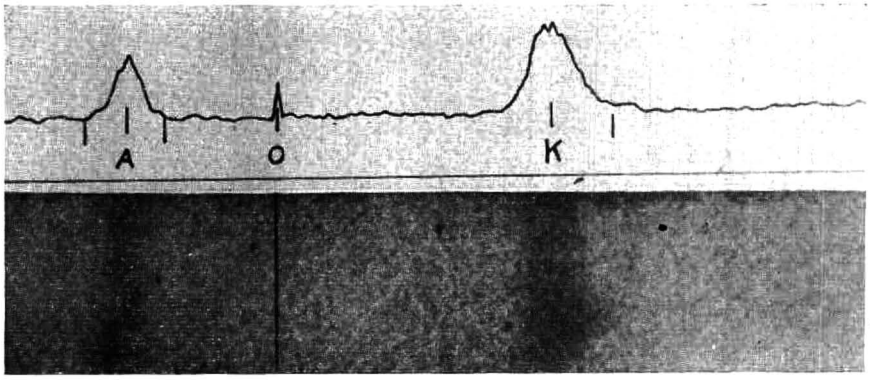


Fig. 6. Electrophoretic migration of CoEDDHA. B-2 buffer, pH 8.6; 15 mA for 7 h. A = Dextrose; O = Origin; K = CoEDDHA; AO = —25.5 mm; OK = +46.7 mm; AK = +72.2 mm.

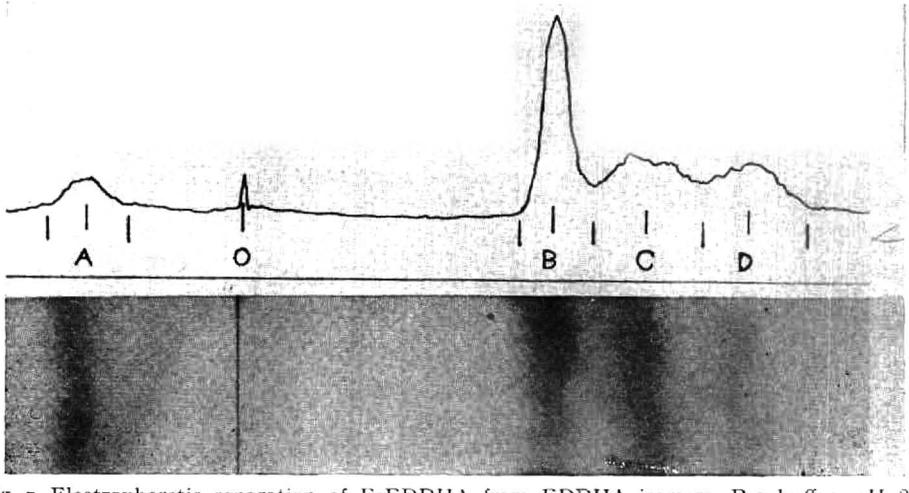


Fig. 7. Electrophoretic separation of FeEDDHA from EDDHA isomers. B-2 buffer, pH 8.6; 15 mA for 7.5 h. A = Dextrose; O = Origin; B = FeEDDHA; C = EDDHA′; D = EDDHA″; AO = —28.3 mm; OB = +52.4 mm; OC = +67.6 mm; OD = +84.8 mm; AB = +80.7 mm; AC = +95.9 mm; AD = +113.1 mm.

TABLE I

ELECTROPHORETIC MIGRATIONS OF EDDHA METAL CHELATES

M_1, M_2 and M_3 are the differential migrations of the isomers of the chelate stated.

Chelate	*Differential migration (mm)*			*Constant current (mA)*	*Time (h)*	*Sample concn./strip (μg)*
	M_1	M_2	M_3			
FeEDDHA	+77.0	—	—	15	7	20
CoEDDHA	+72.2	—	—	15	7	20
CuEDDHA	+89.9	+103.4	—	15	7	20
ZnEDDHA	+59.1	+129.8	—	15	7	20
EDDHA	+95.9	+113.1	—	15	7.5	20
EDDHA	+47.1	+63.0	+71.6	50	3	100

Acknowledgements

The authors thank the Geigy Chemical Corporation, Ardsley, New York, for its financial and material support of this project.

Department of Agricultural Chemistry and Soils, College of Agriculture, University of Arizona, Tucson, Ariz. (U.S.A.)

T. W. McCREARY
R. H. MAIER
B. D. KNEZEK

[1] H. KROLL, M. KNELL, J. POWERS AND J. SIMONIAN, *J. Am. Chem. Soc.*, 79 (1957) 2024.
[2] D. P. RYSKIEWICH AND G. BOKA, *Nature*, 193 (1962) 472.
[3] D. G. HILL-COTTINGHAM, *J. Chromatog.*, 8 (1962) 261.
[4] *Model R Paper Electrophoresis System Instruction Manual, RIM-5*, Beckman/Spinco Div., Palo Alto, Calif.
[5] W. HOYLE AND T. S. WEST, *Talanta*, 3 (1959) 49.
[6] R. M. C. DAWSON, D. C. ELLIOT AND K. M. JONES, (Editors), *Data for Biochemical Research*, Clarendon Press, Oxford, 1959.

Received October 7th, 1963

J. Chromatog., 14 (1964) 544–549

The detection of carboxylic acids on paper chromatograms by means of the dimethylglyoxime–nickel biuret reaction

Most of the known procedures for the detection of acidic spots on paper chromatograms suffer from the disadvantage that either the coloured spots tend to change colour or fade rapidly, or that their initial differentiation from the background is lost as the latter changes colour on storage. The change of background colour may occur within a few minutes (*e.g.* starch–iodide–iodate[1]) or after a few days (*e.g.* acid–base indicators).

Recent studies in this Department have necessitated comparisons over a period of several months of paper chromatograms of carboxylic acids. A method which overcomes the above disadvantages, producing pink-red spots on a white background, stable on paper chromatograms for at least twelve months when stored in the dark, has now been developed.

This method is based on a test devised by FEIGL[2] for acidic substances in solution using the spotting plate technique. For the detection of acidic spots on paper, however, it has been found more expedient to reverse FEIGL's order of application of the reagents, and to increase the sensitivity of the test by adding less alkali in the preparation of the nickel biuret solution than is recommended. To stabilise the colour developed excess reagents are removed from the paper by means of an ethanol–ammonia solution.

Experimental

Reagents

(*1*) *Dimethylglyoxime solution.* 1 % (w/v) solution in 95 % ethanol.

(*2*) *Alkaline nickel biuret solution.* Nickel sulphate heptahydrate (1 g) is dissolved in distilled water (50 ml) and biuret added (1 g), the mixture being warmed to dissolve the biuret. Sodium hydroxide (1 *N*, 10 ml) is added and the mixture allowed to stand

for 30 min before being filtered to remove precipitated nickel hydroxide. A clear, pale amber-coloured solution is obtained. The delay of 30 min between the addition of sodium hydroxide and filtration of the mixture is essential so that the maximum sensitivity is obtained during subsequent use. This reagent should be freshly prepared.

(3) *Ethanol–ammonia solution*. Ammonium hydroxide (0.88 sp.gr., 5 ml) is added to aqueous ethanol (1:1, v/v, 1 l).

Procedure

Any suitable solvent system may be used for developing and separating the carboxylic acid mixture on paper chromatograms. The author has normally used either *n*-butanol–formic acid–water (4:1:5, v/v)[3] or diethyl ether–acetic acid–water (13:3:1, v/v)[4] run in a descending direction on Whatman No. 1 paper. After development, the paper is first allowed to dry in air and is then well steamed to remove all traces of any remaining acidic solvent (formic or acetic acid).

The chromatogram is dipped through a trough containing the dimethylglyoxime solution, allowed to dry partially, and is then sprayed with the alkaline nickel biuret solution. The acids appear as pink to red spots (nickel dimethylglyoxime) on a colourless background, the maximum colour intensity being achieved after 1–2 min. After this time, the chromatogram is washed for 2 min in a large photographic developing dish containing 400–500 ml of the ethanol–ammonia solution, with occasional rocking of the dish. The ethanol–ammonia is then replaced by fresh solution and the paper washed for a further minute, after which time it is blotted between sheets of clean filter paper and dried in a current of warm air.

Discussion

The method has been applied with consistently satisfactory results to the detection of citric, malic, malonic, succinic and fumaric acids in a study of tobacco leaf metabolism. Amounts of the order of 10 μg of each acid are very readily detected.

The possibility of using this reagent for the quantitative estimation of carboxylic acids on paper chromatograms is being currently investigated.

The use of this reagent can very probably be extended to the detection of other classes of acidic compounds, for example phenols, sulphonic acids, etc.

Acknowledgements

The author wishes to thank Dr. B. C. PLATT for originally suggesting the use of the nickel biuret reagent, and the Directors of the Imperial Tobacco Company Limited for permission to publish this note.

Research Department of The Imperial Tobacco Co. (of Great Britain and Ireland), Ltd., Bristol (Great Britain) E. SAVORY

[1] I. SMITH, *Chromatographic Techniques*, Heinemann, London, 1958, p. 209.
[2] F. FEIGL, *Spot Tests*, Vol. 2, *Organic Applications*, Elsevier, Amsterdam, 1954, p. 330.
[3] J. W. H. LUGG AND B. T. OVERELL, *Australian J. Sci. Res.*, 1 (1948) 98.
[4] F. W. DENISON AND E. F. PHARES, *Anal. Chem.*, 24 (1952) 1628.

Received November 1st, 1963

A new solvent system for paper chromatographic separation of glucuronic and galacturonic acids*

In connection with our studies on the chemical composition of basement membrane mucopolysaccharide[1], we developed a new solvent system for the separation of glucuronic and galacturonic acids. Other solvents reported in the literature[2,3] proved unsatisfactory.

The new solvent mixture was made up of analytically pure acetone, ethanol, isopropyl alcohol and 0.05 *M* borate buffer of pH 10.0[4] in the proportion of 3:1:1:2 by volume. Chromatographic data obtained with the above solvent are compared with those of three other solvents in Table I. In these experiments aqueous solutions of the hexuronic acids and their mixtures were applied on Whatman No. 1 paper and chromatographed for 24 h at room temperature in a descending manner. The spots were

Fig. 1. Paper chromatographic separation of glucuronic and galacturonic acids. Solvent: acetone–ethanol–isopropyl alcohol–borate buffer, pH 10.0 (3:1:1:2). 1 = galacturonic acid; 2 = glucuronic acid; 3 = mixture.

* These studies were supported by funds from the University of Michigan Cancer Research Institute, Project 62, American Cancer Society.

TABLE I

COMPARATIVE STUDIES WITH VARIOUS SOLVENTS

Solvent system	Distance travelled from origin in 24 h (cm)	
	Glucuronic acid	Galacturonic acid
Acetone–ethanol–isopropyl alcohol–borate buffer, pH 10.0 (3:1:1:2)	23.7	19.7
tert.-Amyl alcohol–isopropyl alcohol–water (4:1:2)[2]	1.7	1.5
n-Butyl alcohol–acetic acid–water (4:1:2)[2]	13.6	13.2
n-Butyl acetate–acetic acid–butanol–methanol–water (3:2:2:1:1)[3]	11.3	10.5

located on air-dried paper strips with a silver nitrate reagent[5]. Fig. 1 illustrates the separation obtained with the new solvent.

Department of Pathology, The University of Michigan, Ann Arbor, Mich. (U.S.A.)

H. MUKERJEE*
J. SRI RAM

[1] H. MUKERJEE, J. SRI RAM AND G. B. PIERCE, Jr., *Federation Proc.*, 23 (1964) 235.
[2] B. RADHAKRISHNAMURTHY AND G. S. BERENSON, *Arch. Biochem. Biophys.*, 101 (1963) 360.
[3] H. MASAMUNE AND M. MAK, *Tohoku J. Exptl. Med.*, 55 (1951) 299, quoted in E. A. KABAT AND M. M. MAYER, *Experimental Immunochemistry*, 2nd Ed., C. C. Thomas, Springfield, Ill., 1961, p. 525.
[4] C. LONG (Editor), *Biochemist's Handbook*, Van Nostrand Co., Inc., New York, 1961, p. 40.
[5] W. E. TREVELYAN, D. P. PROCTOR AND J. S. HARRISON, *Nature*, 166 (1950) 444.

Received November 12th, 1963

* Elsa U. Pardee Foundation Postdoctoral Fellow.

The paper chromatography of aliphatic sulphonates

Information on the chromatographic behaviour of the aliphatic sulphonic acids and their salts is limited to data for the naturally occurring taurine[1–5] and isethionic acid (2-hydroxyethanesulphonic acid)[3,7], for cysteic acid[1–6], to which cysteine and cystine are often converted during protein degradation studies, and homocysteic acid[1,5,6]. Taurine, cysteic acid and homocysteic acid are readily detected with ninhydrin, and taurine also by reaction with *o*-phthalaldehyde[8], but no simple specific colour reactions for aliphatic sulphonates as a group are available. Aromatic sulphonates, on the other hand, may be detected on chromatograms viewed under U.V. light[9,10] or after being sprayed with Pinacryptol Yellow[11]. In the present communication some methods of possible value in the detection of aliphatic sulphonates have been examined, and chromatographic data for thirteen of these compounds in seven solvent systems are presented.

Materials and methods

With the exception of methanesulphonic acid, ethanesulphonic acid, cysteic acid and taurine, the sulphonic acids examined were obtained synthetically as their sodium salts. These were converted to the corresponding ammonium salts prior to chromatography by treatment of 2 drops of 1 % (w/v) aqueous solutions with 0.25 g of Dowex 50 X 2 resin (NH_4^+ form). Ascending chromatography was carried out at 20° on acetic acid-washed Whatman No. 1 papers, using 10 μg of each salt. Acid washing of the paper greatly improved the definition of the spots subsequently obtained and eliminated background coloration.

Reagents used for the detection of sulphonates

Acid–base indicators. Indicators have previously been used to locate aromatic sulphonic acids on chromatograms[12]. Of a number of indicators tested in the present experiments the most satisfactory was bromocresol green, 0.5 % (w/v) in ethanol, used in the form of a spray. All the ammonium sulphonates were detected with this reagent, but the corresponding sodium salts were not located with accuracy.

Barium chloride–sodium rhodizonate. This reagent has been used in the detection of sulphate ions[13,14] and its possible use to locate sulphonates was therefore examined. Chromatograms were first sprayed with 0.025 % (w/v) aqueous sodium rhodizonate and then sprayed lightly with 10 % (w/v) aqueous barium chloride. Sodium sulphate and ammonium sulphate both appeared as white spots on a yellow background after the initial rhodizonate spray, the barium chloride improving the detection by turning the background pink. Applying the reagents in the reverse order, as described by other workers[13,14], yielded less distinct spots. This reagent did detect sodium and ammonium sulphonates on chromatograms in the majority of solvents used, but it was not sufficiently sensitive (limit of detection 25–50 μg) to warrant general application. No spots were obtained with papers run in pyridine-containing solvents.

Pinacryptol Yellow. Aromatic sulphonates have been found to give yellow, orange or brown spots on a green-yellow background when sprayed with 0.1 % (w/v) aqueous Pinacryptol Yellow followed by examination under U.V. light[11]. This reagent was tested with a range of aliphatic sulphonates. It was found preferable to view chromatograms in reflected U.V. light. The aliphatic sulphonates tested gave fairly distinct light spots on a darker background. As in the case of the previous reagent, however, this method was not sufficiently sensitive for routine detection.

Silver fluoresceinate. POLLARD, NICKLESS AND BURTON[15] have described an effective reagent for anions, including sulphate and sulphamate. This was tested on chromatograms of the aliphatic sulphonates. The reagent consisted of a mixture of one part 10 % (w/v) aqueous silver nitrate and 5 parts 0.2 % (w/v) sodium fluoresceinate in absolute ethanol, prepared immediately before use and sprayed on to chromatograms. Sulphate and all the aliphatic sulphonates tested appeared immediately as pale yellow spots on a salmon-pink background, the spots darkening on exposure to light for some hours. The chromatograms were not permanent owing to eventual darkening of the background. This reagent enabled 1–5 μg of the sulphonates to be detected satisfactorily.

Effect of heat. When dried at 80°, chromatograms which had been developed in solvents containing phenol or mesityl oxide gave brown spots corresponding to the

positions of the sulphonate ions. This charring reaction was also obtained with papers developed in any of the other solvents used if they were dried at 120° or above, but under these conditions location by charring is an unspecific method of detection, being given also by a variety of other compounds[16,17].

Results

Of the above reagents employed, the most suitable for use with aliphatic ammonium sulphonates were bromocresol green and silver fluoresceinate, and R_F values obtained with these reagents were identical. Both were used in the chromatography of ammonium sulphate, sulphamate and thirteen aliphatic sulphonates in the following seven solvent systems:

I. *tert.*-Butanol–formic acid (90 %)–water (16:1:4, v/v/v).
II. Acetone–formic acid (90 %)–water (16:1:3, v/v/v).
III. Mesityl oxide–formic acid (90 %)–water (25:25:12, v/v/v).
IV. Phenol–formic acid (90 %)–water (75:1:25, v/v/v).
V. Ethanol–aqueous ammonium acetate (1 *M*, pH 7.3) (5:2, v/v).
VI. Ethyl acetate–acetic acid–water (3:1:1, v/v/v).
VII. Ethanol–ammonium hydroxide (sp. gr. 0.88)–water (20:1:4, v/v/v).

The summarized results are given in Table I, each value quoted being the average of a minimum of six values. Certain of the solvent systems, notably I, II and VI, gave conveniently wide ranges of R_F values as illustrated by the data for the two alkanesulphonic acids and their bromo-, phenyl- and disulphonic acid derivatives.

TABLE I

R_F VALUES OF AMMONIUM SULPHATE, SULPHAMATE AND SOME ALIPHATIC AMMONIUM SULPHONATES

*Ammonium salt**	$R_F \times 100$						
	Solvent						
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
Sulphate	14	15	62	18	19	6	7
Sulphamate	22	26	61	23	44	13	34
Methanesulphonate	34	35	69	41	68	17	61
Bromomethanesulphonate	59	53	70	44	72	37	64
Iodomethanesulphonate	57	56	71	48	70	39	63
Phenylmethanesulphonate	67	59	80	65	80	56	71
Ethanesulphonate	64	42	68	56	73	20	67
2-Bromoethanesulphonate	70	65	77	54	77	34	72
Isethionate	33	34	64	40	66	14	55
2-Phenylethanesulphonate	72	65	82	69	83	61	74
Taurine	22	17	58	40	51	12	40
Sulphoacetate	27	32	63	20	28	13	19
Methionate (methanedisulphonate)	10	7	61	7	32	5	22
2-Sulphopropionate	34	35	68	27	35	14	29
Ethane-1,2-disulphonate	14	14	66	7	45	5	39

* 10 μg of each salt used on ascending chromatograms of acid-washed Whatman No. 1 paper at 20°.

All solvents tested, however, whether basic or acidic, placed the sulphonates used in approximately the same order on the basis of their R_F values.

Acknowledgements

Thanks are expressed to Dr. A. K. MILLS for his interest and to the Directors of Arthur Guinness Son & Co. (Dublin) Ltd. for permission to publish this work.

Arthur Guinness Son & Co. (Dublin) Ltd., St. James's Gate, Dublin (Ireland)

C. M. COYNE
G. A. MAW

[1] C. E. DENT, *Biochem. J.*, 43 (1948) 169.
[2] H. M. WINEGARD, G. TOENNIES AND R. J. BLOCK, *Science*, 108 (1948) 506.
[3] V. F. COTTY, S. M. HENRY AND J. D. HILCHEY, *Contrib. Boyce Thompson Inst.*, 19 (1958) 379.
[4] D. MARGOLIS AND R. H. MANDL, *Contrib. Boyce Thompson Inst.*, 19 (1958) 509.
[5] P. J. PETERSON AND G. W. BUTLER, *J. Chromatog.*, 8 (1962) 70.
[6] I. SMITH, in R. J. BLOCK, E. L. DURRUM AND G. ZWEIG (Editors), *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press Inc., New York, 1958, p. 135.
[7] J. D. WELTY, W. O. READ AND E. H. SHAW, *J. Biol. Chem.*, 237 (1962) 1160.
[8] G. CURZON AND J. GILTROW, *Nature*, 173 (1954) 314.
[9] G. SPENCER AND V. NIELD, *Chem. Ind. (London)*, (1956) 922.
[10] G. v. HORNUFF AND E. WEINHAUS, *J. Chromatog.*, 8 (1962) 90.
[11] J. BORECKÝ, *J. Chromatog.*, 2 (1959) 612.
[12] A. G. LONG, J. R. QUAYLE AND R. J. STEDMAN, *J. Chem. Soc.*, (1951) 2197.
[13] D. P. BURMA, *Anal. Chim. Acta*, 9 (1953) 513.
[14] J. J. SCHNEIDER AND M. L. LEWBART, *J. Biol. Chem.*, 222 (1956) 787.
[15] F. H. POLLARD, G. NICKLESS AND K. W. C. BURTON, *J. Chromatog.*, 8 (1962) 507.
[16] J. M. HAIS AND K. MACEK, *Handbuch der Papierchromatographie*, VEB G. Fischer Verlag, Jena, 1958, p. 609.
[17] J. LATINÁK, *Collection Czech. Chem. Commun.*, 24 (1959) 922.

Received December 9th, 1963

J. Chromatog., 14 (1964) 552–555

The chromatography of some cations on paper impregnated with ammonium phosphotungstate

Several reports have been published recently on the use of paper impregnated with inorganic ion exchangers for the separation of various cations. ALBERTI AND GRASSINI[1,2] separated alkali metals and alkaline earths using zirconium phosphate impregnated paper and studied the sorption of some cations [Cd(II), Bi(II), Cu(II), Zn(II)] as a function of the pH value. This impregnation has been used for the separation of microgram amounts of Ca, Sr and Ba from each other, and Cs from the alkaline earths[3]. Salts of heteropolyacids have also been used as cation exchangers. Micro amounts of Li, Na, K, Rb and Cs have been separated on paper treated with ammonium 12-molybdatophosphate[4], and this has also been used for the separation of some fission products (^{137}Cs, ^{90}Sr, ^{90}Y, ^{144}Ce)[5]. In this paper the sorption and the separation of ^{86}Rb, ^{134}Cs, ^{89}Sr and ^{91}Y on paper impregnated with ammonium phosphotungstate (NH_4PW) is described. The sorption of caesium on this heteropolyacid has already been studied by KRTIL AND KOUŘIM[6] under static conditions, and the dynamic sorption and elution of caesium and rubidium was reported by KRTIL[7] using columns of NH_4PW precipitated on asbestos.

Experimental

The radioisotopes used were 10^{-3} *M* solutions of ^{134}Cs and ^{86}Rb with carrier-added and carrier-free ^{89}Sr and ^{91}Y.

The pH values of the solutions were measured with a pH meter (Radiometer, Copenhagen). Chromatograms were run by the descending techniques. Strips of NH_4PW-paper were spotted with 2 λ of the solution containing the radioisotopes under study, and were developed with NH_4NO_3 solutions of various concentrations and pH values. The positions of the spots were detected by measuring the activity of the radioisotopes with a G.M. counter with a mica window. The strip is passed under the counter at a distance of 5 mm from the window. All chemicals used were of analytical grade purity.

Preparation of ion exchange papers. Strips (3 × 45 cm) of Whatman No. 4 paper were drawn through the solution of NH_4NO_3, the excess liquid was absorbed by several sheets of filter paper and the strips were dried in the air at room temperature. The dry strips were thoroughly sprayed with a solution of phosphotungstic acid (HPW) and again dried. The unprecipitated ions were removed by dipping the strips into distilled water for 10 min and the prepared papers were dried between two sheets of filter paper.

Results and discussion

ALBERTI[2] *et al.* derived the following relation between the concentration of NH_4^+ ions in the solvent and R_M values for paper impregnated with zirconium phosphate:

$$-x \log [NH_4^+] = R_M + \text{const.} \quad (1)$$

where x is the valence of an adsorbed ion.

From our data it is evident that this relation is valid also for the paper impregnated with NH_4PW and for ^{134}Cs and ^{89}Rb radioisotopes. The R_M values, as a function of the concentration of the eluate NH_4NO_3, are presented in Tables I, II and Fig. 1.

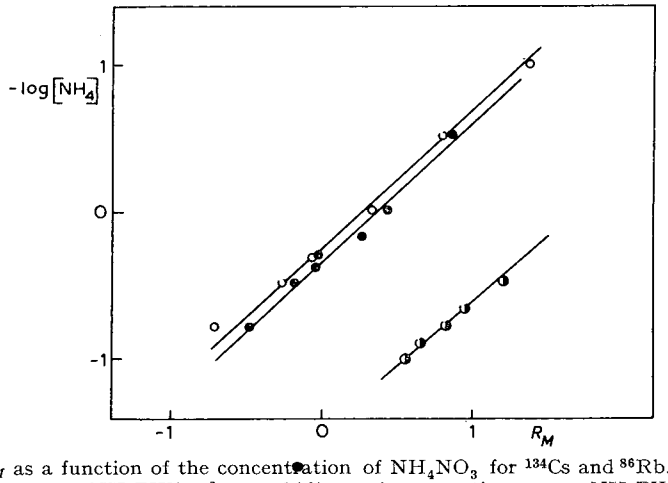


Fig. 1. R_M as a function of the concentration of NH_4NO_3 for ^{134}Cs and ^{86}Rb. ○ rubidium – impregnation 1.9 mg NH_4PW/cm^2; ● rubidium – impregnation 4.3 mg NH_4PW/cm^2; ◑ caesium – impregnation 1.9 mg NH_4PW/cm^2.

TABLE I

R_F AND R_M VALUES FOR RUBIDIUM IN NH_4NO_3 OF VARIOUS CONCENTRATIONS

Concentration of NH_4NO_3 (*M*)	0.1	0.3	1.0	2.0	3.0	6.0
R_F	0.04	0.15	0.31	0.53	0.65	0.84
R_M	1.38	0.81	0.34	—0.05	—0.26	—0.72

If the values of —log $[NH_4^+]$ are plotted against R_M, they fall on a straight line with a slope 1. The pH of the eluate was 5 ± 0.2. The accuracy of the determination of R_F values was ± 0.05.

The validity of the relation (1) has also been confirmed for different amounts of

TABLE II

R_F AND R_M VALUES FOR CAESIUM IN NH_4NO_3 OF VARIOUS CONCENTRATIONS

Concentration of NH_4NO_3 (*M*)	3.0	4.5	6.0	8.0	10.0
R_F	0.06	0.10	0.13	0.18	0.22
R_M	1.20	0.95	0.83	0.66	0.55

rubidium spotted on the starting point. From Table III and Fig. 2 it may be seen that relation (1) is valid for amounts exceeding 0.06 mg rubidium spotted in a volume of 1 λ on the starting point. The R_M values were constant up to this limit and decrease for $m_{Rb} > 0.06$ mg. In all these experiments the same conditions of elution were maintained (0.2 *M* HNO_3 + 1 *M* NH_4NO_3). From Fig. 1 it is also evident that the

TABLE III

VERIFICATION OF EQUATION (1) WITH VARIOUS AMOUNTS OF RUBIDIUM SPOTTED ON THE STARTING POINT

Log m_{Rb}	R_F	R_M	R_F	R_M
—3.28	0.32	0.32	0.27	0.43
—2.84	0.30	0.37	0.25	0.48
—2.54	0.28	0.41	0.26	0.45
—2.36	0.32	0.32	0.25	0.48
—1.52	0.35	0.27	0.27	0.43
—1.07	0.32	0.32	0.29	0.39
—0.77	0.40	0.18	0.32	0.33
—0.29			0.43	0.12
0.01	0.56	—0.10	0.43	0.12
0.19	0.62	—0.21	0.49	0.02
0.31	0.63	—0.23	0.56	—0.11
Density of impregnation mg/cm_2	1.9		4.3	

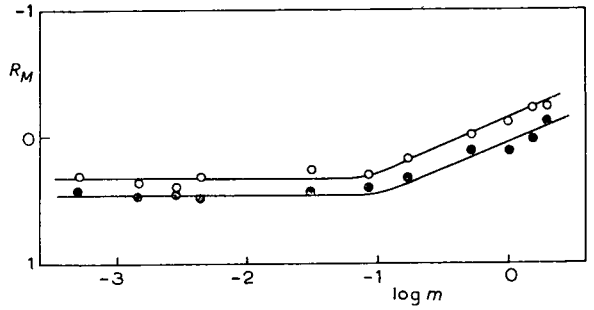


Fig. 2. R_M as a function of the amount of Rb spotted on the starting point (elution conditions constant). O impregnation 1.9 mg NH_4PW/cm^2; ● impregnation 4.3 mg NH_4PW/cm^2.

influence of the density of impregnation was small, and the differences in $(R_F)_{Rb}$ were very small for densities of 1.9 mg/cm² and 4.3 mg/cm², respectively.

The influence of the pH of the eluate on the $(R_M)_{Rb}$ has also been investigated. In Fig. 3 it is shown that the R_M values were independent of pH for pH $>$ 0.4 and decrease from pH $<$ 0.4.

The sorption and the elution of ^{89}Sr and ^{91}Y has only been investigated in the range necessary for the successful separation of these two elements.

The R_F of yttrium is zero in the concentration range 1–6 M NH_4NO_3 at pH = 5, while strontium moves with the liquid front with 1 M NH_4NO_3 or 1 M HNO_3 ($(R_F)_{Sr} = 1$).

The following optimum conditions for the separation of rubidium, caesium, strontium and yttrium on ammonium phosphotungstate paper have been chosen, based on the behaviour of these elements.

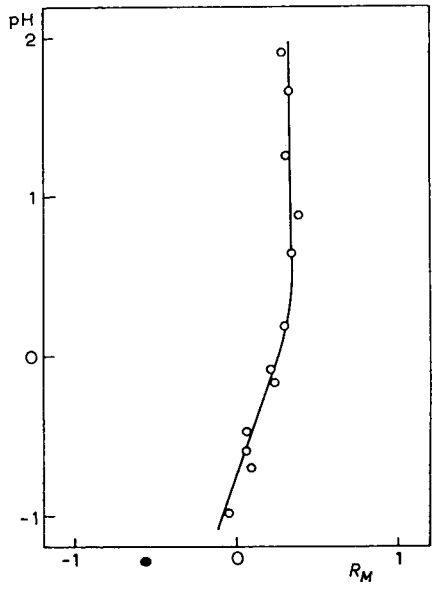


Fig. 3. The influence of the pH value of the eluate on $(R_M)_{Rb}$.

Separation of caesium and rubidium. Good separation of microamounts and macroamounts (0.5 mg Rb and 1.5 mg Cs) has been obtained by elution with 3 *M* NH_4NO_3 (see Fig. 4).

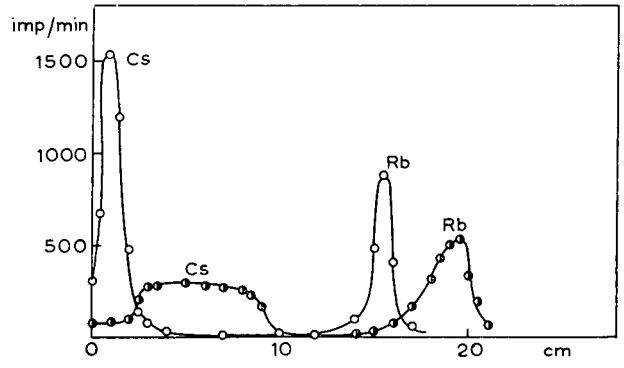


Fig. 4. Separation of Rb and Cs with 3 *M* NH_4NO_3. ○ – microamounts of Rb and Cs; $(R_F)_{Cs}$ = 0.05; $(R_F)_{Rb}$ = 0.60. ◑ – macroamounts, 0.5 mg Rb and 1.5 mg Cs.

Separation of strontium and yttrium. ^{89}Sr and ^{91}Y have been separated by elution with 1 *M* NH_4NO_3 at pH = 5. Under these conditions $(R_F)_Y$ = 0 and $(R_F)_{Sr}$ = 1.

Separation of caesium and yttrium. Solutions of 1 *M* HNO_3 or over, are the best eluants for the separation of caesium and yttrium; $(R_F)_{Cs}$ = 0, $(R_F)_Y$ = 1.

Separation of rubidium and yttrium. Elution with 2 *M* NH_4NO_3, pH = 5, has been used for the separation of these two elements. Under these conditions $(R_F)_Y$ = 0 and $(R_F)_{Rb}$ = 0.53.

Separation of rubidium, caesium, strontium and yttrium. Fig. 5 shows the separation of these four radioisotopes. The chromatogram has been spotted with the mixture of the solution ^{86}Rb, ^{134}Cs, ^{89}Sr, ^{91}Y chlorides and developed for 90 min with

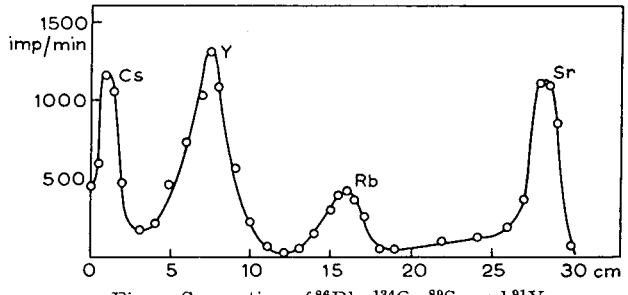


Fig. 5. Separation of ^{86}Rb, ^{134}Cs, ^{89}Sr and ^{91}Y.

1.5 *M* NH_4NO_3 + 0.05 *M* HNO_3. Under these conditions rubidium and strontium are separated from a mixed zone of caesium and yttrium. To achieve further separation of caesium and yttrium the mixture of 1.5 *M* NH_4NO_3 and 2 *M* HNO_3 has been used as eluant. After 70 min development a good separation was obtained ($(R_F)_{Cs}$ = 0.04; $(R_F)_Y$ = 0.25; $(R_F)_{Rb}$ = 0.53; $(R_F)_{Sr}$ = 0.94.

Acknowledgements

The authors wish to thank Prof. Dr. V. MAJER for his interest in this work and Ing. J. KRTIL C.Sc. for critical discussion.

Department of Nuclear Chemistry,
Faculty of Technical and Nuclear Physics,
Prague (Czechoslovakia)

J. PRÁŠILOVÁ
F. ŠEBESTA

[1] G. ALBERTI AND G. GRASSINI, *J. Chromatog.*, 4 (1960) 83.
[2] G. ALBERTI, G. GRASSINI AND F. DOBICI, *J. Chromatog.*, 8 (1962) 103.
[3] J. M. PEIXOTO CABRAL, *J. Chromatog.*, 4 (1960) 86.
[4] G. ALBERTI AND G. GRASSINI, *J. Chromatog.*, 4 (1960) 423.
[5] H. SCHROEDER, *J. Chromatog.*, 6 (1961) 361.
[6] J. KRTIL AND V. KOUŘIM, *J. Inorg. Nucl. Chem.*, 12 (1960) 367.
[7] J. KRTIL, *J. Inorg. Nucl. Chem.*, 19 (1961) 298.

Received December 10th, 1963

J. Chromatog., 14 (1964) 555–560

Glyoxal-bis-(2-hydroxyanil), ein Reagens für papierchromatographisch getrennte Kationen

Metallionen bilden mit Glyoxal-bis-(2-hydroxyanil)* (GBHA) tieffarbige Metallkomplexe und Salze[1–4]. Die tiefe Farbe der Komplexe hat zu empfindlichen qualitativen Nachweisreaktionen und quantitativen Bestimmungsmethoden für einige Metallionen geführt[2,5,6]. Während im sauren pH-Bereich wegen der Umlagerungsreaktion des Komplexbildners nur einige wenige Metallionen selektiv gebunden werden[3], bilden im alkalischen Milieu nahezu alle Metallionen Komplexe oder Salze. Diese starke pH-Abhängigkeit der Komplexbildung legt nahe, das GBHA als allgemein anwendbares, aber durch die Abstufung der Komplexbildung doch selektives Sprühreagens für papierchromatographisch getrennte Kationen zu verwenden.

Deshalb wurde zunächst untersucht, wie sich die Komplexe und damit die tiefen Farben beim Besprühen mit GBHA-Lösungen bei unterschiedlichen pH-Werten bilden. Die Ergebnisse sind in Tabelle I wiedergegeben. Vier Papiere, auf denen die Metallsalze aufgetragen waren, wurden mit 5 *N* Salszäure besprüht, weil die Laufmittel bei der Papierchromatographie der anorganischen Kationen fast immer Säure enthalten. Anschliessend wurde getrocknet. Die Papiere wurden dann entweder mit Wasser (a), einer Acetatpufferlösung pH 6 (b) oder einer *N*/10 Natronlauge (c) besprüht. Darauf wurde getrocknet und mit einer 1 %igen methanolischen GBHA-Lösung behandelt. Unter (d) sind die Farben angeführt, die beim Besprühen mit einer methanolischen Lösung von 1 % GBHA und 3 % Kalilauge erhalten wurden. Wie aus früheren Untersuchungen zu erwarten war, werden unter den Bedingungen (d) die meisten Metallionen angezeigt. Aus diesem Grund wird (d) als universelles Reagens vorgeschlagen. Die Nachweisgrenze der Metallionen mit GBHA liegt bei 1–5 γ. Beim Cadmium wird sogar eine Nachweisgrenze von 0.5 γ erreicht.

* Glyoxal-bis-(2-hydroxyanil) kann von der Firma Merck, Darmstadt bezogen werden.

TABELLE I

FARBEN BEIM BESPRÜHEN MIT GBHA-LÖSUNG BEI VERSCHIEDENEN pH-WERTEN

Ion	*Sprühreagens**			
	(a)	*(b)*	*(c)*	*(d)*
Ag	—	bräunlich	bräunlich	grau
Al	—	—	—	—
AsO_4^{3-}	—	weiss	weiss	hellgrau
Ba	—	—	rosa	rot
Be	—	weiss	weiss	—
Bi	—	—	—	blau
Ca	schwach rosa	schwach rosa	rosa	rot
Ce	blaugrau	braun	braun	braun
Cd	—	—	rosa	violett
Co	—	violett	violett	violett
Cr^{3+}	—	graublau	graublau	gelb
CrO_4^{2-}	gelb**	gelb**	gelb**	gelb**
Cu	—	gelbgrün	blaugrün	blauviolett
Fe^{3+}	gelb**	schwach braun	schwach braun	schwach braun
Hg_2^{2+}	—	—	braun	orange
Hg^{2+}	—	—	braun	braun
La	—	rotviolett	rotviolett	rotviolett
Li	—	—	—	rotorange
Mg	—	—	—	rot
Mn	—	—	braun	braun
MoO_4^{2-}	—	—	—	weiss
Ni	—	schwach rosa	rosa	rot
Pb	—	—	grau	blau
Sb	—	—	—	schwach lila
Sn^{2+}	—	weiss	weiss	graublau
Sr	—	—	schwach rosa	rot
Tl^{+}	—	—	—	rot
TiO^{2+}	—	—	—	gelblich
UO_2^{2+}	—	—	—	graublau
VO_4^{3-}	—	—	schwach braun	ocker
V^{4+}	—	—	—	braun
WO_4^{2-}	schwach gelb	schwach gelb	schwach gelb	schwach braun
Zn	—	—	rosa	rotviolett
ZrO^{2+}	—	—	blassviolett	blassviolett

* Vgl. Beschreibung im Text.
** Eigenfarbe

Um nun die Leistungsfähigkeit des Reagenses bei der Papierchromatographie zu prüfen, untersuchten wir neben einem Gemisch aus Nickel(II)-, Kobalt(II)-, Kupfer(II)-, Cadmium- und Zinkionen die verschiedenen analytischen Gruppen (Tabelle II). Es wurde nach der aufsteigenden Methode chromatographiert. Hierbei zeigte sich für die Salzsäuregruppe, dass bei Verwendung des Laufmittels *n*-Butanol–Pyridin–Wasser nach MURTHY und Mitarb.[7] die einwertigen Silber- und Quecksilberionen nach 6 und 14 St. Laufzeit Schwänze bildeten. Durch das GBHA-Reagens lassen sich die Kationen trotzdem leicht durch ihre charakteristische Farbe unterscheiden.

Bei den Bemühungen um eine gute papierchromatographische Auftrennung der Kationengemische, konnte mit *n*-Butanol–5 *N* Salzsäure–Aceton–Acetylaceton,

Bei den Bemühungen um eine gute papierchromatographische Auftrennung der Kationengemische, konnte mit *n*-Butanol–5 *N* Salzsäure–Aceton–Acetylaceton,
125:25:150:1, V/V) eine gute Trennung für die zweiwertigen Ionen des Nickels,

TABELLE II

R_F-WERTE DER METALLIONEN VERSCHIEDENER ANALYTISCHER GRUPPEN

Ion	*R_F-Wert*	*Farbe*	*Laufmittel*	*Laufzeit (Std.)*
Pb	0.02	graublau	*n*-Butanol–Pyridin–Wasser	6
Tl^+	0.07	rot	(60:4:36, V/V)	
Ag	0.35*	grau	nach MURTHY und Mitarb.[7].	
Hg_2^{2+}	0.87*	orange		
Ni	0.02	rot		$4^1/_2$
Co	0.12	violett		
Cu	0.48	blauviolett		
Cd	0.89	violett		
Zn	0.95	rotviolett		
Pb	0.28*	graublau	*n*-Butanol–5 *N* Salzsäure–Aceton–Acetylaceton (125:25:150:1, V/V)	6
Cu	0.41	blauviolett		
Cd	0.74	violett		
Bi	0.87	blau		
Hg^{2+}	0.94	braun		
Mo	0.21*	weiss		14
As	0.70	hellgrau		
Sb	0.87	schwach lila		
Sn^{2+}	0.97	graublau		
Ni	0.09	rot	Aceton–20% Salzsäure	$2^1/_4$
Mn^{2+}	0.31	braun	(88:12, V/V)	
Co	0.57	rotviolett	nach KAKÁC[8]	
Zn	0.94	rotviolett		
Ba	0.14	rot	Isopropanol–Pyridin–Wasser–Eisessig	3
Sr	0.26	rot	(8:8:4:1, V/V)	
Ca	0.47	rot	nach GORDON UND HEWEL[9]	
Mg	0.76	rot		

* Schwanzzone vom Start, R_F-Wert bezogen auf die Spitze.

Kobalts, Kupfers, Cadmiums und des Zinks erzielt werden (vgl. Fig. 1 und Tabelle II). Bereits nach 4 St. war die Trennung vollständig. Dieses neue Laufmittel wurde auch erfolgreich bei der Trennung der Kationen der Schwefelwasserstoffgruppe verwendet. Auch hier konnten nach 6 St. Laufzeit die dicht beieinanderliegenden Kationen des Cadmiums, Wismuts und des zweiwertigen Quecksilbers der Kupfergruppe leicht durch die charakteristischen Farben unterschieden werden (vgl. Fig. 2). Ebenso ist eine Trennung und Identifizierung der Ionen der Arsengruppe möglich.

Während für die Ammonsulfidgruppe eine gute Bestimmung der Kationen mit dem GBHA-Reagens und dem von KAKÁC[8] vorgeschlagenen Laufmittel Aceton–Salzsäure bereits nach $2^1/_4$ Stunden erreicht werden konnte, verliefen die Versuche, die Ionen der Urotropingruppe mit einem geeigneten Laufmittel zu trennen, nicht zufriedenstellend. Mit dem von GORDON UND HEWEL[9] angegebenen Laufmittel konnten die Erdalkalien nach dreistündiger Laufzeit als scharfe rote Farbflecke aus dem Gemisch erkannt werden.

Experimenteller Teil

Aufgetragen wurden die Kationen als Nitrate und Chloride in einer Menge von 10–20 γ Metallion auf Schleicher & Schüll Papier 2043 Mgl. Die Kammern waren jeweils mit

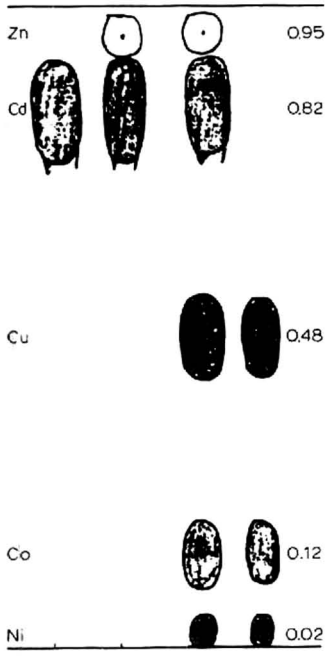


Fig. 1. Chromatogramm der zweiwertigen Ionen des Nickels, Kobalts, Kupfers, Cadmiums und des Zinks. Laufmittel: *n*-Butanol–5 *N* Salzsäure–Aceton–Acetylaceton (125:25:150:1, V/V). Sprühreagens: eine methanolische Lösung von 1 % GBHA und 3 % Kalilauge.

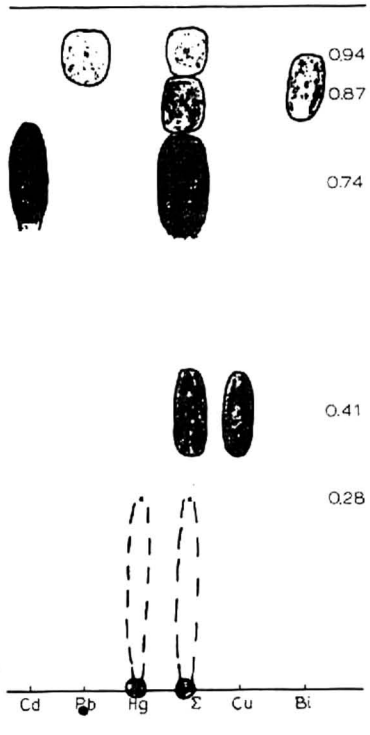


Fig. 2. Chromatogramm der Ionen der Schwefelwasserstoffgruppe. Laufmittel: *n*-Butanol–5 *N* Salzsäure–Aceton–Acetylaceton (125:25:150:1, V/V). Sprühreagens: eine methanolische Lösung von 1 % GBHA und 3 % Kalilauge.

dem Laufmittel gesättigt. Nach dem Trocknen wurde das Chromatogramm mit einer methanolischen Lösung von 1 % GBHA und 3 % Kalilauge besprüht und mit einem Föhn (etwa 50°) getrocknet, die Farbflecke markiert und mit dem Vergleichschromatogramm identifiziert.

Dank

Wir möchten Herrn Prof. Dr. ERNST BAYER für die Aufgabenstellung und seine Unterstützung herzlichst danken. Unser Dank gilt auch Herrn Dr. K. L. HOCK und Herrn R. BACHL für ihre Hilfe.

Chemisches Institut der Universität Tübingen, (Deutschland)

H. G. MÖLLER
N. ZELLER

[1] E. BAYER, *Chem. Ber.*, 90 (1957) 2785.
[2] E. BAYER UND H. MÖLLINGER, *Angew. Chem.*, 71 (1959) 426.
[3] E. BAYER UND G. SCHENK, *Chem. Ber.*, 93 (1960) 1184.
[4] G. SCHENK, *Dissertation*, Technische Hochschule, Karlsruhe, 1961.
[5] D. GOLDSTEIN UND C. STARK-MAYER, *Anal. Chim. Acta*, 19 (1958) 437.
[6] F. UMLAND UND K. U. MECKENSTOCK, *Angew. Chem.*, 72 (1960) 324.
[7] V. MURTHY, V. A. NARAYAN UND M. R. A. RAO, *Current Sci. (India)*, 24 (1955) 158.
[8] B. KAKÁC, in: I. M. HAIS UND K. MACEK (Herausgeber), *Handbuch der Papierchromatographie*, Bd. I, Fischer Verlag, Jena, 1960.
[9] H. T. GORDON UND C. A. HEWEL, *Anal. Chem.*, 27 (1955) 1471.

Eingegangen den 13. Dezember 1963

J. Chromatog., 14 (1964) 560–564

Some observations on iodine vapour as a reagent

In paper-electrophoretic analyses of alkaloids and certain amines, it was found that development by iodine vapour produced more coloured spots than had been expected. Since the spots appeared invariably in the same positions (towards the cathode) when a given technique was employed, paper contaminations were suspected. Indeed, the foreign spots were found to be due to a considerable contamination of the paper with sodium and a minor contamination with magnesium. Previously washed papers did not exhibit these spots in the course of the electrophoretic analysis (Fig. 1).*

The problem was particularly interesting in that it furnished additional data on the development of alkali metals. (Another striking reaction of alkali metals has recently been pointed out by GROSS[1] who claims that potassium, sodium, lithium, magnesium and calcium give positive ninhydrin reactions on paper.)

Attention was directed to the use of iodine vapour by BRANTE[2] and MARINI-BETTÒLO *et al.*[3]. MARINI-BETTÒLO[4] recommends it as a universal developer for paper chromatography, pointing out, amongst other things, though without giving details,

* To confirm the assumption of sodium and magnesium contamination, we determined the migration ratio of sodium and magnesium relative to potassium (SCHIER[5], GROSS[6]) by our paper electrophoretic technique. The values obtained (Na: 0.77, Mg: 0.50) agreed with the foreign spots produced on the paper. The identity of sodium was also confirmed separately by development with magnesium uranyl acetate.

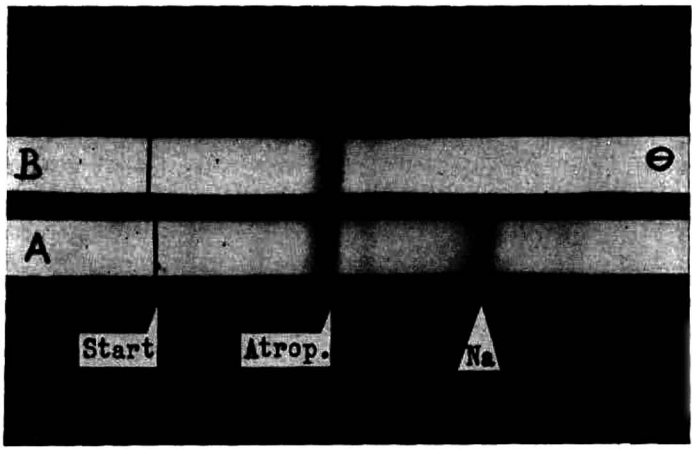


Fig. 1. Electropherograms of atropine salt after migration on unwashed (A) and washed (B) papers and development with iodine vapour. The pale strip between those of atropine and sodium is that of magnesium.

that iodine vapour permits even inorganic ions to be developed. We have, therefore, examined the sensitivity of the iodine development of potassium, sodium, lithium and magnesium after paper electrophoretic migration. The equipment is a simplified version of Grassmann-Hannig's apparatus. Potential gradient: 11 V/cm. Current intensity: 1–1.5 mA/cm. Electrolyte: 0.2 *M* ammonium bicarbonate. Paper: Schleicher & Schüll 2043/b, 10 × 250 mm, washed. Washing: soaking in water for 1 h, followed by flushing and drying. Such paper will, after the migration is finished, exhibit only very pale magnesium spots. Application: 30 mm from the centre towards the anode. Period: 30 min.

After the migration is finished, the dried paper strips were developed for 30 min at room temperature in an iodine atmosphere. The sensitivity of development was 1 μg for potassium, sodium and lithium, and approximately 1 μg for magnesium. The spots are brown in colour. Like MARINI-BETTÒLO[4], we also found that the sensitivity of development increases with the period of iodine vapour treatment. Furthermore, it was found that using 0.75 *M* formic acid as electrolyte, the above ions could be developed, though with reduced sensitivity.

In order to study the development procedure, model experiments were carried out without electrophoretic migration. Amounts of aqueous solutions of various sodium salts corresponding to 10 μg sodium were applied to the washed paper. The paper was treated with iodine vapour after the spots had dried. It was found that the sodium salts of strong mineral acids (chloride, nitrate, sulphate) gave spots of very pale colour, whereas the sodium salts of weak acids (carbonate, bicarbonate, borate, acetate, formate) and sodium hydroxide (*i.e.* the base-hydrolysing salts) developed an intensive brown colour. This observation, too, appears to confirm MARINI-BETTÒLO's finding[4], that the possibility of iodine development is due to the changes produced by the compound in the cellulose structure and not to the compound itself. It may be reasonably assumed that such changes resulting in an increase in the iodine-binding capacity of the cellulose are due only to base-hydrolysing sodium salts.

Finally, it should be noted that a considerable sodium contamination of chro-

matographic filter papers will not be apparent and cause an unexpected spot during the iodine development unless the following technique of application is employed.

The solution of the test substance is placed on the dry paper strip; the paper is then immersed in the electrolyte and wetted in such a way that the start section is left dry. The soaked strip is placed in the apparatus and wetting of the start section occurs by absorption of liquid from the two electrode vessels. As the liquid fronts approach the start line, the sodium, and other alkaline contaminations, otherwise uniformly distributed throughout the paper, are washed out and driven towards the start line. The sodium content of the 20–25 mm wide strip section (left dry during the initial absorption) thus becomes concentrated on the start line in the form of a narrow strip. With this technique, it is always advisable to use washed paper if iodine-vapour development is to be applied.

The appearance of alkali metal spots should, however, be expected even on washed paper and using any type of technique involving iodine development in cases where the test solution contains alkali metals either in the form of contaminations or components.

Acknowledgements are due to Prof. Dr. ANTAL VÉGH for his valuable aid and advice in our work.

Institute of Pharmaceutical Chemistry,
Budapest University of Medicine,
Budapest (Hungary)

MIHÁLY TAKÁCS

[1] D. GROSS, *J. Chromatog.*, 10 (1963) 233.
[2] G. BRANTE, *Nature*, 163 (1949) 651.
[3] G. B. MARINI-BETTÒLO AND S. GUARINO, *Experientia*, 6 (1950) 309.
[4] G. B. MARINI-BETTÒLO, *Rend. Ist. Super. Sanità*, 17 (1954) 471.
[5] O. SCHIER, *Angew. Chem.*, 68 (1956) 63.
[6] D. GROSS, *J. Chromatog.*, 10 (1963) 221.

Received October 21st, 1963

Technique et résultats d'une méthode d'électrophorèse en gel de gélose avec dépôt direct de l'échantillon protéique, à la surface du gel

Depuis la description de l'électrophorèse en milieu gélifié par GORDON *et al.*[1] et de l'immuno-électrophorèse par GRABAR ET WILLAMS[2], d'intéressantes modifications ou diverses améliorations ont été apportées aux méthodes d'électrophorèses en gel de gélose[3–10].

Nous nous proposons de montrer certains avantages d'une technique d'électrophorèse avec dépôt direct de l'échantillon à la surface du gel. Le réservoir de départ étant supprimé, la couche de gel ne présente aucune interruption: il en résulte une distribution plus uniforme du champ électrique lors de la migration, et un meilleur enregistrement photométrique après coloration. Cette méthode donne de bons résultats pour l'immuno-électrophorèse[11].

Technique

Le gel utilisé est formé de 0.8 % de gélose soigneusement purifiée et d'un tampon véronal pH 8.6: soit tampon Michaelis dilué au demi, soit tampon Durrum additionné de lactate de calcium[12]. Le gel est coulé sur des plaques de verre 24 h avant l'électrophorèse, de façon à obtenir une très légère déshydratation qui favorisera par la suite l'absorption du produit déposé. Les plaques de verre utilisées mesurent généralement 18 × 3 cm. La quantité de gélose coulée sur chaque plaque est alors de 9 ml. On peut aussi utiliser des plaques de plus petites dimensions, mais au dessous de certaines limites, elles se prêtent moins bien à l'enregistrement photométrique.

Le sérum, ou tout autre échantillon convenable, est déposé directement à la surface du gel au moyen d'une micropipette graduée, à extrémité coudée, et à pointe fine et rodée[11]. La longueur du trait de dépôt est d'environ 15 mm. Il est possible de répartir de façon homogène 1 à 10 μl du produit à analyser en ayant soin de laisser absorber progressivement le liquide.

La migration electrophorétique s'effectue dans une enceinte à atmosphère humide et réfrigérée, selon un dispositif analogue à ceux antérieurement décrits.

Les protéines sont ensuite fixées par immersion des plaques dans une solution d'acide acétique à 2 %. La dessication du gel est assurée par application sur chaque plaque d'une feuille de papier filtre[5].

La coloration est réalisée selon la méthode d'URIEL[10] au moyen d'une solution de noir amide ou de nigrosine à 1‰ en tampon acétate. Pour l'enregistrement photométrique, et l'évaluation quantitative en pourcentages des diverses fractions sériques, on utilise le récent photomètre enregistreur A.P.E.L.A.B. MONTAGNE qui se prête bien à la lecture des électrophorèses en milieu gélifié.

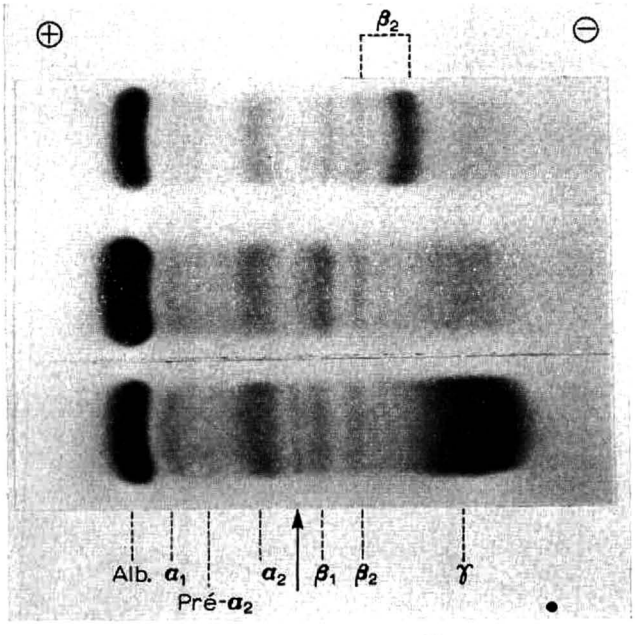
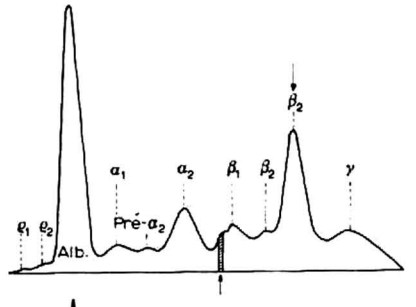
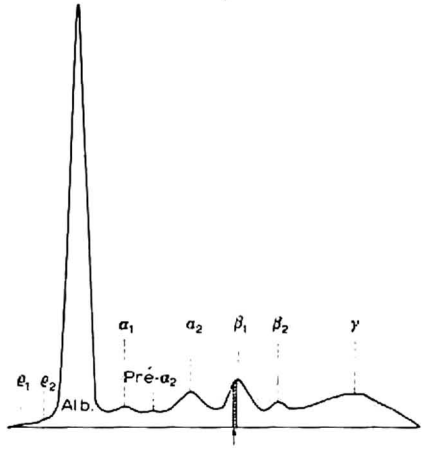


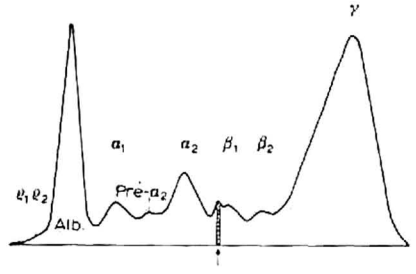
Fig. 1.



Hyper-β_2-globulinémie	
albumine	36 %
α_1-globulines	4 %
pré-α_2-globulines	2 %
α_2-globulines	10 %
β_1-globulines	6 %
β_2-globulines	5 % / 25 %
γ-globulines	12 %



Sérum normal	
albumine	58 %
α_1-globulines	4 %
pré-α_2-globulines	3 %
α_2-globulines	8 %
β_1-globulines	9 %
β_2-globulines	4 %
γ-globulines	14 %



Hyper-γ-globulinémie	
albumine	20 %
α_1-globulines	4 %
pré-α_2 globulines	3 %
α_2-globulines	8 %
β_1-globulines	5 %
β_2-globulines	4 %
γ-globulines	56 %

Fig. 2.

Résultats

L'absorption directe par le gel du produit à analyser ne semble pas apporter de modifications notables dans le comportement des protéines (exception faite évidemment pour les très faibles quantités restant, au niveau du dépôt, à la surface du gel). La tache de dépôt est peu marquée. Dans certaines conditions de concentration en protéines, on peut obtenir, après coloration par la nigrosine, un enregistrement photométrique permettant une évaluation quantitative.

D'une façon générale, la séparation des différentes fractions protéiques du sérum apparaît nette et régulière: β_1 ,β_2- et γ-globulines sont bien distinctes. Ce fait semble intéressant pour une étude quantitative des diverses variétés électrophorétiques d'anticorps.

La Fig. 1 montre trois électrophorégrammes de sérums humains pratiqués dans les conditions suivantes: gélose en tampon Michaelis dilué, dépôt de 5 μl de sérum, coloration par le noir amide. Au centre: sérum normal; en haut: hyper-β_2-globulinémie; en bas: hyper-γ-globulinémie. La flèche indique le trait de dépôt. On remarque l'existence d'une fraction intermédiaire entre α_1 et α_2 (désignée pré-α_2 sur les Figs. 1 et 2).

La Fig. 2 présente les tracés électrophorétiques de ces trois sérums et les valeurs en pourcentages des différentes fractions (gélose en tampon Durrum-lactate, dépôt: 3 μl, coloration: nigrosine).

Cette méthode fournit aussi de bons résultats pour la séparation des protéines sériques de lapin, de rat et de cheval. Elle est également utilisable pour l'étude des extraits cellulaires et de certains produits microbiens.

Remerciements

Nous remercions vivement Mesdames C. GAILLARD et J. CHAPELLIER de leur collaboration.

Laboratoire de la M.G.E.N., Institut Prophylactique, Paris (France)*

PH. GOULLET
H. KAUFMANN

1 A. H. GORDON, B. KEIL, K. SEBESTA, O. KNESSEL ET F. SORM, *Collection Czech. Chem. Commun.*, 15 (1950) 1.
2 P. GRABAR ET C. A. WILLIAMS, *Biochim. Biophys. Acta*, 10 (1953) 193.
3 A. BUSSARD ET D. PERRIN, *J. Lab. Clin. Med.*, 46 (1955) 689.
4 J. J. SCHEIDEGGER, *Intern. Arch. Allergy Appl. Immunol.*, 7 (1955) 103.
5 J. URIEL ET J. J. SCHEIDEGGER, *Bull. Soc. Chim. Biol.*, 37 (1955) 165.
6 N. RESSLER ET B. ZAK, *Clin. Chim. Acta*, 1 (1956) 392.
7 K. V. GIRI, R. NATH ET H. GRIKANTIAN, *J. Indian Inst. Sci.*, 39 (1957) 224.
8 C. WUNDERLY ET V. BUSTAMANTE, *Klin. Wochschr.*, 35 (1957) 758.
9 R. J. WIEME, *Clin. Chim. Acta*, 4 (1959) 317.
10 J. URIEL, dans P. GRABAR ET P. BURTIN (Éditeurs), *Analyse immuno-électrophorétique*, Masson, Paris, 1960, p. 33.
11 PH. GOULLET, *Experientia*, 20 (1964) 49.
12 W. B. YEOMAN, *Clin. Chim. Acta*, 4 (1959) 523.

Reçu le 9 décembre, 1963

* 36, Rue d'Assas, Paris, VI°.

An improvement in the method for separation of certain nucleosides, nucleotides and creatine phosphate*

The Dowex-1 formate column[1] is probably the most widely used method for separation of nucleotides. There are, however, two distinct drawbacks to this method: (a) the low pH of the eluent induces breakdown of acid-labile compounds such as creatine phosphate and (b) the high pK values (over 9) for anionic dissociation of most of the nucleosides leads to their simultaneous elution from the column.

* Supported by USPHS Grants HE-06031-02 and HE-06304-02.

MARTONOSI[2] introduced the use of Dowex-1 in the bicarbonate form for the separation of nucleotides. With this system ion exchange chromatography can be carried out at a near neutral pH. However, with the gradient elution employed with this method, only poor separation of substances with low affinity for the resin, like nucleosides and nucleoside monophosphate, can be achieved. The shape of the concentration gradient of the eluent in this system increases exponentially, reaching the concentration of the solution in the reservoir asymptotically. Moreover, the need to replace the solution in the reservoir for each change in concentration gradient makes it impossible to carry out uninterrupted chromatographic runs. The present study represents an attempt to improve chromatographic separation of nucleotides and related compounds by use of the variable gradient device of PETERSON AND SOBER[3] without subjecting the column to low pH.

Dowex-1 bicarbonate resin (X10; 200–400 mesh) was prepared in the manner described by MARTONOSI[2]. The resin was poured as a thin aqueous slurry into a glass tube of 1.0 cm I.D. to a height of 12 cm. A mixture of a number of nucleosides and nucleotides was put on the column in approximately 10 ml at a rate of 0.6 ml/min. Four consecutive chambers of a nine chamber "Varigrad"* were used; they were filled with the following solutions respectively: water, 5 % potassium bicarbonate, water, and 13 % potassium bicarbonate. Constant flow from the first chamber of the Varigrad to the column was maintained at 0.4 ml/min with a Buchler pump. The

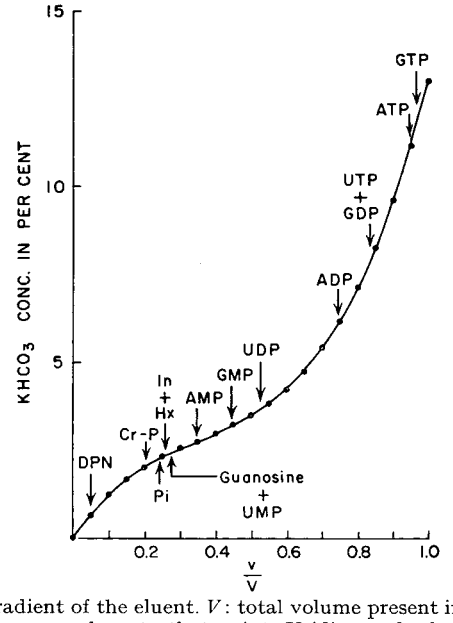


Fig. 1. Concentration gradient of the eluent. V: total volume present in the system. v: the volume of the eluent which has emerged up to that point. Uridine and adenosine appear in the breakthrough. Points of elution indicated by arrows. DPN = diphosphopyridine nucleotide; Cr-P = creatine phosphate; Pi = inorganic phosphate; In = inosine; Hx = hypoxanthine; UMP = uridylic acid; AMP = 5′-adenylic acid; GMP = guanylic acid; UDP = uridine diphosphate; ADP = adenosine diphosphate; UTP = uridine triphosphate; GDP = guanosine diphosphate; ATP = adenosine triph sphate; GTP = guanosine triphosphate.

* Buchler Instruments Inc.

eluate was collected in 5 ml fractions with a Gilson Medical Electronics automatic fraction collector and the optical density of the eluate at 260 mμ was continuously recorded with a Gilford multiple sample absorbance recorder positioned between the column and the fraction collector. Fig. 1 depicts the concentration gradient of the system and the points where added substances were eluted. A chromatogram of a mixture of certain nucleotides and their derivatives (4–6 μmoles of each) is schematically presented in Fig. 2. As can be seen from these figures, the method is satisfactory

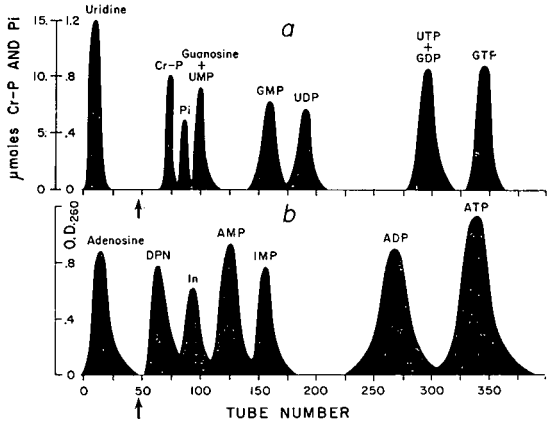


Fig. 2. Schematic tracings of the chromatograms of various nucleosides and nucleotides, creatine phosphate and inorganic phosphate. Ordinate: optical density of the nucleoside and nucleotide fractions at 260 mμ, and the concentration of Cr-P and Pi in μmoles/fraction. Abscissa: number of 5 ml fractions collected. IMP = inosine triphosphate. For other abbreviations, see Fig. 1.

for the separation of some physiologically important compounds found in muscle (Fig. 2b). Although complete separation of DPN and creatine phosphate, and of inosine and inorganic phosphate could not be achieved by this method, each of these compounds can be determined in the presence of the other by properly selected methods.

Department of Physiology,
Western Reserve University School of Medicine,
Cleveland, Ohio (U.S.A.)

SHOICHI IMAI*
ROBERT M. BERNE**

[1] R. B. HURLBERT, H. SCHMITZ, A. F. BRUMM AND V. R. POTTER, *J. Biol. Chem.*, 209 (1954) 23.
[2] A. MARTONOSI, *Biochem. Biophys. Res. Commun.*, 2 (1960) 12.
[3] E. A. PETERSON AND H. A. SOBER, *Anal. Chem.*, 31 (1959) 857.

Received December 20th, 1963

* Permanent address: Department of Pharmacology, School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan.
** USPHS Research Career Awardee Grant No. HE-K6-9593.

Book Reviews

Gas Phase Chromatography, by R. KAISER, translated by P. H. SCOTT, Butterworths, London, 1963.

Vol. 1: *Gas Chromatography*, price 42 s.

Vol. 2: *Capillary Chromatography*, price 35 s.

Vol. 3: *Tables for Gas Chromatography*, price 40 s.

This is the latest addition to the already numerous monographs on gas chromatography in the English language, being the revised translation of a three-volume work originally published in German. These books are directed to lecturers, chemists and students and the aim of the author is to render them familiar with this highly effective branch of analytical physical chemistry.

The first volume deals very shortly with the theory of gas chromatography, quite extensively with various items of apparatus and with the determination and the evaluation of qualitative and quantitative analytical results. The second volume is devoted to the remarkable possibilities of capillary column chromatography. It consists of three sections: the first one deals with the theory of capillary gas partition chromatography, the second with the methods and apparatus and the third with the applications in qualitative and quantitative analysis. The experimental problems are treated quite extensively and a large amount of information is supplied for each part of apparatus and for any operation related to this technique.

The third volume is a supplement to volume I and II. It is a collection of tables of column packings for given separations, of liquid phases and adsorbents, of solid supports, of retention volumes and of substance specific correction factors for quantitative analysis with different detectors by using various gases as carrier gas. Formulae, currently used in gas chromatography, are included as well as other tables of various types. Anyone engaged in problems of analytical research by means of gas chromatography will find this volume extremely valuable, because of the large amount of data concisely reported. From this aspect this volume has unique features.

In all the volumes the material is pleasantly presented and clearly and neatly described so that anyone who wishes to have a fairly thorough picture of different subjects related to gas chromatography may be well informed. Only minor remarks might be raised: as an example the electron capture detector is only shortly mentioned and a statement such as the impossibility for a chemist to build an ionization current amplifier might be not true now-a-days when a large variety of simple schemes are readily available.

The academic worker or anyone who is contemplating research on a scientific stand point might be not quite satisfied with this work because of the shortness of the theoretical part but the analytical and practical chemist will greatly enjoy to have these volumes on his desk.

A. LIBERTI (Naples)

Ionenaustauscher, von F. HELFFERICH, Band I, *Grundlagen, Struktur, Herstellung, Theorie*, Verlag Chemie GmbH, Weinheim/Bergstr., 1959, viii + 520 Seiten.

Überarbeitete englische Ausgabe: *Ion Exchange*, Mac Graw-Hill Book Company, Inc., 1962, ix + 624 Seiten.

Das zu besprechende Buch ist ein Standardwerk für alle theoretischen Fragen des Ionenaustausches. Es behandelt zunächst kurz Struktur, Wirkungsweise, Aufbau, Eigenschaften und Herstellung von Ionenaustauschern und dann ausführlich Kapazität, Gleichgewichte, Kinetik, elektrochemische Eigenschaften, Ionenaustauschermembranen, Ionenaustauscherpackungen, Verhalten in nicht wässrigen und gemischten Lösungsmitteln, Ionenaustauscher als Katalysatoren sowie Redoxaustauscher (Elektronenaustauscher).

Zwischen Lehrbuch und Monographie stehend, behandelt es den Stoff von einem einheitlichen Gesichtspunkt unter Verzicht auf manchen verwirrenden historischen Ansatz. Andererseits ist die Darstellung sehr eingehend und mit etwa 2500 Literaturzitaten versehen. Die ersten hundert Seiten eignen sich auch als erste Einführung, das letzte Kapitel als solche für Redoxaustauscher.

Der guten theoretischen Behandlung sind jeweils qualitative Übersichten vorangestellt und Zusammenfassungen angefügt. Sie lesen sich durch den lebendigen Stil besonders flüssig und erleichtern das Verständnis sehr. Bei der mathematischen Behandlung der thermodynamischen Kapitel möchte man allerdings an einzelnen Stellen den Autor zu dem in der englischen Ausgabe bereits eingeschlagenen Weg einer weiteren Straffung und mathematischen Präzisierung bestärken. Zur Kinetik des Ionenaustausches hat der Autor im Institut von BONHOEFFER selbst wesentliche Beiträge geliefert und neuerdings die Methode des Ligandenaustausches entdeckt. Eingehend werden auch chromatographische Vorgänge in Säulen theoretisch behandelt. Dem Ziel des Buches entsprechend, sind die experimentellen Abschnitte nur kurz. Gelegentliche kleinere Mängel darin scheinen in der englischen Ausgabe bereits beseitigt zu sein. Zu der sicher bald notwendigen Neuauflage wird man sich vielleicht kleine gesonderte Kapitel über den Molekülbau unter Berücksichtigung neuer Ergebnisse der U.R.-Spektroskopie, anorganische Ionenaustauscher und Chelataustauscher wünschen dürfen. Das letzte Kapitel sollte man wohl besser mit "Redoxaustauscher" überschreiben und in Redoxite und Redox-Ionenaustauscher unterteilen, da der Name "Elektronenaustauscher" nicht zutreffend ist. Es ist richtig, dass Redox-Harze häufig nur wenige Redoxumwandlungen reversibel ausgehalten haben. Es sei jedoch der Hinweis gestattet, dass in unserem Laboratorium neuerdings ein solches Harz bereits 250–300 mal zu analytischen Anwendungen hintereinander reduziert und oxydiert wurde.

Die Leistung des Autors ist am besten zu würdigen durch den Hinweis auf die ausgesprochene Grenzstellung des Ionenaustausches zwischen der physikalischen Theorie der Oberflächenadsorption, der physikalisch-chemischen Theorie der Membrangleichgewichte und konzentrierten Elektrolytlösungen sowie Polyelektrolyte, den Gelen der Kolloidchemie, den chemischen Modellen Festsäure, Festbase, Festion und unlösliches Fällungsreagens, den Membranen der physiologischen Chemie, dem Ackerboden der Bodenkunde und Hohlraum-Gittern der Kristallographie. Diese zahllosen Arbeiten verarbeitet und unter einen Hut gebracht zu haben, dafür schulden wir dem Autor Dank

Das Buch gibt über alle Fragen der Theorie des Ionenaustauches Auskunft. Es kann weiterhin zur Examensvorbereitung und als Nachschlagewerk für ein Ionenaustauscherpraktikum verwendet werden. Die Anschaffung ist in jedem Arbeitskreis, der sich mit Ionenaustauschern beschäftigt, notwendig und darüber hinaus jeder chemischen Institutsbibliothek zu empfehlen.

B. SANSONI (Marburg/Lahn)

J. Chromatog., 14 (1964) 573–574

Vitamin-Bestimmungen, by R. STROHECKER AND H. M. HENNING, edited by E. Merck AG, Darmstadt, published by Verlag Chemie GmbH, Weinheim/Bergstr., 365 pages, price DM 42.—.

This book is the second edition of a collection of the analytical methods used in the vitamin analysis laboratory of E. Merck, AG, Darmstadt. Its main difference from the earlier edition and similar books lies in the extensive application of thin-layer chromatography in the various quantitative methods. The book is illustrated with superb colour plates showing thin-layer chromatograms and apparatus. Polarograms and absorption spectra are also presented in excellent multicolour plates and the printing and lay-out makes it easily one of the best produced books which the reviewer has seen for some time.

It will be equally invaluable to workers in the field of vitamins and to chemists who want to incorporate chromatographic methods into quantitative determinations and are looking for accurately described examples.

J. Chromatog., 14 (1964) 574

Techniques in Protein Chemistry, by J. LEGGETT BAILEY, Elsevier Publishing Co., Amsterdam, 1962, x + 310 pages, price 60s, Dfl. 30.

Of the eleven chapters of this book six deal with separation methods and the rest with chemical topics such as disulphide bonds, selective cleavage of peptide chains, N-terminal sequence and C-terminal sequence determinations, etc.

The separation methods considered of importance by the author are paper chromatography, high and low voltage electrophoresis, ion exchange chromatography of amino acids and peptides as well as of proteins, gel filtration and adsorption chromatography.

Most chapters assume a certain familiarity with the techniques and thus hardly overlap with monographs dealing with the techniques proper. They do contain a good selection of data and much working detail without completely covering the literature.

The author succeeded admirably in the writing of a small book to serve biochemists as an introduction to protein chemistry and its techniques.

J. Chromatog., 14 (1964) 574

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

SZASZ, G.: (Fifteen years of paper chromatography). *Gyogyszereszet*, 3 (1959) 296–300; *C.A.*, 58 (1963) 1889h.

WOHLLEBEN, G.: Chromatographie-Geräte. *Glas-Instr.-Tech.*, 6 (1962) 381; *ibid.*, 7 (1963) 1, 43 and 221 — a review of apparatus for column, paper and thin-layer chromatography; explanation of terms used in chromatography.

2. FUNDAMENTALS, THEORY AND GENERAL

SOCZEWINSKI, E., WAKSMUNDSKI, A. AND MACIEJEWICZ, W.: (Paper-chromatographic method for the determination of suitable solvent systems for countercurrent distribution. II. Experimental part). *Bull. Acad. Polon. Sci., Ser. Sci. Chim.*, 10 (1962) 125–130; *C.A.*, 58 (1963) 1950c.

3. TECHNIQUES I

BORST-PAUWELS, G. W. F. H. AND DE MOTS, A.: Strip-transfer method applicable in two-dimensional paper chromatography. *Experientia*, 19 (1963) 51–53.

CHEBOTAREV, A. I.: (Pigments for control of chromatographic separation of a mixture of colorless substances). *Tr. Vologodsk. Molochn. Inst.*, No. 44 (1961) 370–377; *C.A.*, 58 (1963) 9401f — fourteen dyes were selected as indicators for *n*-butanol–acetic acid–water system.

MÖHRLE, H. AND ROTH, H. J.: Zur Spezifität der TTC-Reaktion. 1. Mitt. TTC-Reaktion tertiärer Phenacylamine. *Arch. Pharm.*, 296 (1963) 811–819.

PALEČEK, M. AND DEYL, Z.: (Apparatus for paper chromatography). *Czech. Pat.* 103,588, May 15, 1902, Appl. Feb. 21, 1959; *C.A.*, 58 (1963) 7378e.

7. PHENOLS

DEVI, A., MUKUNDAN, M. A., PATIL, F. V. AND SARKAR, N. K.: Isolation and characterisation of a polyhydroxy compound in adult *Tribolium confusum* Duval. *Experientia*, 19 (1963) 404–405.

HALMEKOSKI, J. AND NISSEMA, A.: (Relation between the structure and R_m values of related catechols and their partition coefficients between water and aliphatic ethers). *Suomen Kemistilehti*, 35B (1962) 188–191; *C.A.*, 58 (1963) 1947h.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

POWERS, J. J., SOMAATMAJDA, D., PRATT, D. E. AND HAMDY, M. K.: Anthocyanins. II. Action of anthocyanin pigments and related compounds on the growth of certain microorganisms. *Food Technol.*, 14 (1960) 626–632; *C.A.*, 58 (1963) 9437g.

SUNDT, E. AND SACCARDI, A.: Dünnschichtchromatographie und Papierchromatographie der gesmacksgebenden Bestandteile der Vanille. *Food Technol.*, 16, No. 4 (1962) 89–91; from *Z. Lebensm. Untersuch. -Forsch.*, 120 (1963) 59.

9. OXO COMPOUNDS

FIGURSKI, R. AND MICHALSKA, Z.: (Chromatographic method for the detection of vanillin). *Przemysl Spozywczy*, 16 (1962) 41–43; *C.A.*, 58 (1963) 10394b.

ONODA, R.: (Paper chromatography of aldehydes and ketones). *Nippon Kagaku Zasshi*, 82 (1961) 1721–1724; *C.A.*, 58 (1963) 9609f.

ROTHE, M. AND THOMAS, B.: Aromastoffe des Brotes Versuch einer Auswertung chemischer Geschmackanalysen mit Hilfe des Schwellenwertes. *Z. Lebensm. Untersuch. -Forsch.*, 119 (1963) 302.

10. CARBOHYDRATES

HALLER, H.: (Paper chromatography of urine sugar). *Aerztl. Forsch.*, 16 (1962) 350–356; *C.A.*, 58 (1963) 2636b.

INSHAKOV, M. D. AND SIVKOVA, A. P.: (Improved paper-chromatographic method for analysis of carbohydrates in spent sulfite liquors and prehydrolyzates). *Nauchn. Tekhn. Inform. Tsentr. Inst. Nauchn.-Tekhn. Inform. Bumazhn. i Derevoobrabat. Prom. Tsellyulozn.-Bumazhn., Gidrolizn. i Lesokhim. Prom., Sb.*, No. 2 (1961) 40–48; *C.A.*, 58 (1963) 9322b.

MOMOSE, T. AND MAKAMURA, M.: Organic analysis. XXIX. A new spraying agent for reducing sugars on paper chromatograms. *Talanta*, 10 (1963) 115–116; *C.A.*, 58 (1963) 9398a — 3,6-dinitrophthalic acid.

TAKI, M.: Chromatography of starches. I. Paper chromatography of starches. *Nippon Nogeikagaku Kaishi*, 33 (1959) 216–218; *C.A.*, 58 (1963) 2559f.

TAKI, M.: Chromatography of starches. II. Quantitative paper chromatography of starches. *Nippon Nogeikagaku Kaishi*, 33 (1959) 218–220; *C.A.*, 58 (1963) 2559h.

TAKI, M.: Chromatography of starches. III. Separation and determination of amylose in starches by means of paper chromatography. *Nippon Nogeikagaku Kaishi*, 33 (1959) 245–249; *C.A.*, 58 (1963) 2560d.

TAKI, M.: Chromatography of starches. IV. Determination of the amylose contents of various starches by paper chromatography. *Nippon Nogeikagaku Kaishi*, 33 (1959) 445–448; *C.A.*, 58 (1963) 2560f.

TAKI, M.: Chromatography of starches. V. A new method for paper chromatography of starches. *Nippon Nogeikagaku Kaishi*, 33 (1959) 448–452; *C.A.*, 58 (1963) 2560g.

TAKI, M.: Chromatography of starches. VI. Amylose content of starches from rice with different maturities. *Nippon Nogeikagaku Kaishi*, 33 (1959) 778–781; *C.A.*, 58 (1963) 2560h.

TAKI, M.: Chromatography of starches. VII. Efficacy of amylose precipitants for the fractionation of amylose and amylopectin. *Nippon Nogeikagaku Kaishi*, 33 (1959) 781–785; *C.A.*, 58 (1963) 2561a.

TIMELL, T. E.: (Enzymic hydrolysis of a 4-O-methylglucuronoxylan from the wood of white birch). *Svensk Papperstid.*, 65 (1962) 435–447; *C.A.*, 58 (1963) 10436a.

WILLIAMSON, A. R. AND ZAMENHOF, S.: Detection and rapid differentiation of glucosamine, galactosamine, glucosamine uronic acid, and galactosamine uronic acid. *Anal. Biochem.*, 5 (1963) 47–50 — indole reagent as a specific spray for 2-amino-2-deoxy sugars.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BERNARD, A. H. AND ROST, H. E.: Analysis of lipids and oxidation products by partition chromatography. Dimeric and polymeric products. *J. Am. Oil Chemists' Soc.*, 39 (1962) 479; *C.A.*, 58 (1963) 2573e.

CONNORS, W. M. AND BOAK, W. K.: Reversed-phase paper chromatography of saturated fatty acids. *Tobacco Sci.*, 7 (1963) 4–6 (publ. in *Tobacco*, 156, No. 2 (1963) 30–32); *C.A.*, 58 (1963) 10498a.

GUTHENBERG, H. AND BECKMAN, I.: Identifizierung von Konservierungsmitteln auf Papierchromatogrammen durch Bestrahlung mit kurzwelligem Ultraviolettlicht. *Z. Lebensm. Untersuch. -Forsch.*, 120 (1963) 461.

IBRAGIMOV, A. P. AND ARIFZHANOV, S.: (Paper chromatographic separation of fatty acids and identification of irradiation products). *Vopr. Sovrem. Fiz. i. Mat., Akad. Nauk. Uz. SSR*, (1962) 105–111; *C.A.*, 58 (1963) 8152g.

KUMAR, S. AND AVENA, R. M.: Paper chromatographic separation of acetyl and β-hydroxybutyryl hydroxamates. *Anal. Biochem.*, 5 (1963) 265–267.

MATIA, E. R.: (Chromatographic analysis of organic acids in *Taraxacum kok-saghyz*). *Bol. Inst. Nacl. Invest. Agron. (Madrid)*, 22, No. 46 (1962) 123–131; *C.A.*, 58 (1963) 3680e.

13. STEROIDS

KLEMPIEN, E. J., VOIGT, K. D. AND TAMM, J.: Conversion of dehydroisoandrosterone in (isolated) dog livers. *Acta Endocrinol.*, 36 (1961) 498–510; *C.A.*, 58 (1963) 3665f.

OERTEL, G. W.: Isolation of 17-hydroxypregnenolone from peripheral human blood plasma. *Acta Endocrinol.*, 37 (1961) 301–304; *C.A.*, 58 (1963) 3664f.

OERTEL, G. W. AND EIK-NES, K.: Isolation of pregnenolone from canine adrenal vein blood. *Acta Endocrinol.*, 37 (1961) 305–308; *C.A.*, 58 (1963) 3664g.

SIMPSON, T. H. AND WRIGHT, R. S.: The characterization of estrogens. *Anal. Biochem.*, 5 (1963) 313–320 — 2,7-dichlorofluorescein used as a non-destructive reagent for phenolic steroids.

WENGLE, B. AND BOSTRÖM, H.: Studies on ester sulphates. 14. The *in vitro* formation of steroid disulphates in rat liver extracts. *Acta. Chem. Scand.*, 17 (1963) 1203–1217.

14. STEROID GLYCOSIDES

KOLOS-PETHES, E.: (Examination of the crude saponins in the roots of *Viola odorata* by paper chromatography). *Gyogyszereszet*, 3 (1959) 473–474; *C.A.*, 58 (1963) 2637b.

WICHTL, M., PEITHNER, G. AND FUCHS, L.: (Paper-chromatographic separation and quantitative determination of cardenolide glycosides of *Convallaria* herb and tincture). *Planta Med.*, 10 (1962) 304–317; *C.A.*, 58 (1963) 10040d.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

NEU, R.: The analysis of washing and cleaning agents. XIX. Distinguishing the types of nitrogen combinations in surface-active substances. *Seifen-Oele-Fette-Wachse*, 88 (1962) 535; *C.A.*, 58 (1963) 10409b.

18. AMINO ACIDS

BENEVENT, M. T., ROQUES, M., TORRESANI, J. AND LISSITZKY, S.: Désiodation enzymatique de la thyroxine et de ses dérives. III. Désiodation photochimique de la *l*-thyroxine en l'absence de tissus. *Bull. Soc. Chim. Biol.*, 45 (1963) 969–987.

FELLEGIOVÁ, M.: Quantitative Bestimmung des *p*-Hydroxybenzoesäureäthylesters mit Hilfe der Papierchromatographie. *Z. Lebensm. Untersuch. -Forsch.*, 120 (1963) 17.

GRAM, H. F., BERRIDE, Jr., B. J., ACTON, E. M. AND GOODMAN, L.: Potential anticancer agents. LXXX. Alkylating agents related to phenylalanine mustard. 6. Enanthiomeric *meta* phenylalanine mustards. *J. Med. Chem.*, 6 (1963) 85–87; *C.A.*, 58 (1963) 8954a.

JORYSCH, D., SARRIS, P. AND MARCUS, S.: (Nachweis von organischen Säuren in Fruchtsäften mittels Papierchromatographie). *Food Technol.*, 16, No. 3 (1962) 90–93; from *Z. Lebensm. Untersuch. -Forsch.*, 120 (1963) 36.

KOCHETKOV, N. K., DEREVITSKAYA, A. V. AND MOLODTSOV, N. V.: (Glycopeptides. III. Synthesis of N-aminoacyl derivatives of amino sugars). *Zh. Obshch. Khim.*, 32 (1962) 2500–2505; *C.A.*, 58 (1963) 9223d.

LAKSHMI, M. S. AND MULKERKAR, L.: Paper chromatographic study of the role of sulphur-containing amino acids in the process of induction in the chick embryo. *Experientia*, 19 (1963) 155–156.

LANDO, L. I.: (Chromatographic investigation of the serum of schizophrenia patients). *Zh. Nevropatol. i Psikhiatr.*, 62 (1962) 1855–1862; *C.A.*, 58 (1963) 10611c.

LUCKNER, M.: Über Bildung und Stoffwechsel von α-N-Acetyl-D-tryptophan und α-N-Acetyl-D-kynurenin bei *Penicillium viridicatum* Westling. *Z. Allgem. Mikrobiol.*, 3 (1963) 93–100.

NJAA, L. R.: Methionine detection on paper chromatograms. *Acta Chem. Scand.*, 17 (1963) 1169–1170 — modified iodoplatinate reagent.

NOFRE, C., CHARRIER, J. P. AND CIER, A.: Étude quantitative de la destinée des acides aminés sous l'effet du radical libre hydroxyle. *Bull. Soc. Chim. Biol.*, 45 (1963) 913–923.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

BAJUSZ, S., LENARD, K., KISFALUDY, L., MEDZIHRADSZKY, K. AND BRUCKNER, V.: (Synthesis of the dodecapeptide derivative for building a corticotropin-active polypeptide. (Preliminary communication)). *Acta. Chim. Acad. Sci. Hung.*, 30 (1962) 239–243; *C.A.*, 58 (1963) 3506e.

FRIED, M. AND PUTNAM, F. W.: Peptide chromatograms of normal human and myeloma γ-globulins. *Biochemistry*, 1 (1962) 983–987; *C.A.*, 58 (1963) 3749d.

MEDZIHRADSZKY, K., BRUCKNER, V., KAJTAR, M., LOW, M., BAJUSZ, S. AND KISFALUDY, L.: (Synthesis of a nonapeptide derivative for building up adrenocorticotropin-active polypeptides). *Acta. Chim. Acad. Sci. Hung.*, 30, No. 1 (1962) 105–108; *C.A.*, 58 (1963) 3505b.

NAGY, D., GATI, L. AND KELLER, G.: Paper chromatography of pregnancy urine. *Acta Endocrinol.*, 36 (1961) 431–437; *C.A.*, 58 (1963) 2617a.

SHIMONISHI, Y., SAKAKIBARA, S. AND AKABORI, S.: Studies on the synthesis of peptides containing glutamine as the C-terminal. I. Protection of amide-nitrogen with xanthyl groups during peptide synthesis. *Bull. Chem. Soc. Japan*, 35 (1962) 1966–1970; *C.A.*, 58 (1963) 9228h.

20. PROTEINS

BERGSTRÖM, S., DRESSLER, F., RYHAGE, L., SAMUELSSON, B. AND SJÖVALL, J.: (Prostaglandin and related factors. VIII. The isolation of two further prostaglandins from sheep prostate glands). *Arkiv Kemi*, 19 (1962) 563–567; *C.A.*, 58 (1963) 3727f.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

GIANNESSI, P.: (Examination of derivatives of thiouracil in feeds). *Ann. Staz. Chim.-Agrar. Sper. Roma, Ser. III, Pubbl.* 190 (1961) 6; *C.A.*, 58 (1963) 3830f.

KLÖCKING, H. P.: Die Verwendung von Niederschlag-Papier bei der papierchromatographischen toxikologischen Barbituratanalyse. *Z. Med. Labortech.*, 2 (1961) 271–274.
KLÖCKING, H. P.: Der chemische Nachweis von Barbituraten auf Papierchromatogrammen. *Z. Med. Labortech.*, 3 (1962) 289–300.
KLÖCKING, H. P. AND JOSCHKO, E.: Modifiziertes Quecksilber(II)-sulfat-Reagents für Barbiturate. *Z. Chem.*, 2 (1962) 350.
LETTRE, H. AND BALLWEG, H.: (Ribosides of some cytotoxic purine derivatives). *Ann.*, 656 (1962) 158–162; *C.A.*, 58 (1963) 2499b.
ŽIVKOV, V. I.: (Free nucleotides in sternal and leg muscles of chicks). *Doklady Akad. Nauk SSSR*, 149 (1963) 453–455.

22. ALKALOIDS

ADAMANIS, F., PAWELCZYK, E. AND PLOTKOWIAKOWA, Z.: (Analysis of decomposition products of Hydergin-type drugs). *Farm. Polska*, 18, No. 8, (1962) 180–183; *C.A.*, 58 (1963) 8852f.
DANILOVIC, M. AND RISTIC, N.: (Separation of opium alkaloids by paper chromatography). *Arhiv Farm. (Belgrade)*, 12, No. 2 (1962) 112–114; *C.A.*, 58 (1963) 10037h.
KOLUSHEVA, A. AND TONCHEVA, P.: (Detection and determination of small amounts of atropine in the presence of morphine). *Farmatsiya (Sofia)*, 11, No. 6 (1961) 13–18; *C.A.*, 58 (1963) 8851d.
NAKAMURA, G. R. AND UKITA, CH.: A study of hydrolysis of heroin by paper chromatography. *J. Forensic Sci.*, 7 (1962) 465–473; *C.A.*, 58 (1963) 3272f.
PINYAZHKO, I. R. M.: (Chromatographic investigation of some alkaloids). *Farmatsevt. Zh. (Kiev)* 17, No. 3 (1962) 26–28; *C.A.*, 58 (1963) 7783g — indole alkaloids.
VISHIN, M. L., SCHRÖTER, H. B. AND MUNSCHE, D.: Die Bestimmung des Damascenins. *Arch. Pharm.*, 296 (1963) 859–860.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

MACRAE, E. K.: Chromatographic identification of porphyrins in several species of *Turbellaria*. *Experientia*, 19 (1963) 77–78.
VEGAS, F. R.: Chromatographic behavior on ion-exchange paper of bilirubin conjugated with sulphate and glucuronide. *Anal. Biochem.*, 5 (1963) 465–470.
WAKSMUNDSKI, A. AND PRZYBOROWSKA, M.: (Chromatographic determination of the content of quinoline bases in stripping oil). *Ann. Univ. Mariae Curie-Sklodowska, Lublin-Polonia, Sect. D*, 16 (1961) (Pub. 1962) 183–187; *C.A.*, 58 (1963) 8823g.

24. ORGANIC SULPHUR COMPOUNDS

BRÄUNIGER, H. AND MOEDE, F.: (Sulfonamide N-glycosides. III. Paper chromatography of sulfonamides and sulfonamide N-glycosides). *Pharm. Zentralhalle*, 101 (1962) 383–389; *C.A.*, 58 (1963) 8855b.
ENOS, H. F. AND FREAR, D. E. H.: Method for the detection of microgram quantities of O,O-dimethyl-S-(N-methylcarbamoylmethyl)phosphorodithioate (dimethoate) in milk. *J. Agr. Food Chem.*, 10 (1962) 477–479; *C.A.*, 58 (1963) 3825b.
FISCHER, W. AND JAEHN, L.: (Paper chromatographic examination of different adhesives of the polychloroprene type). *Adhaesion*, 6 (1962) 609–612; *C.A.*, 58 (1963) 10417h.
SOPHOULIS, E.: Qualitative analysis of some sulfonamides by paper chromatography. *J. Pharm. Sci. U. Arab. Rep.*, 2, No. 1 (1961) 29–34; *C.A.*, 58 (1963) 8854h.
SVENNERHOLM, L.: Chromatographic determination of sulphatides. *Acta Chem. Scand.*, 17 (1963) 1170–1172.

25. ORGANIC PHOSPHORUS COMPOUNDS

UKITA, C. AND HAYATSHU, H.: (Organic phosphates. XVIII. Syntheses of lyxouridine 2',3'-cyclic phosphate and related compounds). *Chem. Pharm. Bull. (Tokyo)*, 9 (1961) 1000–1005; *C.A.*, 58 (1963) 2500c.

26. METALLO-ORGANIC COMPOUNDS

NESMEYANOV, A. N., PEREVALOVA, E. G. AND SHILOVTSEVA, L. S.: (Aminomethylation of phenyl-, tolyl-, and chloroferrocenes). *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1962) 1767–1772; *C.A.*, 58 (1963) 7972c.

27. VITAMINS

HÜTTENRAUCH, R. AND KLOTZ, L.: Vitamintrennungen an Ionenaustauscherpapieren. *Experientia*, 19 (1963) 95–96 — vitamins of the B group.

KLOTZ, L. AND HÜTTENRAUCH, R.: Die Trennung wasserlöslicher Vitamine an Ionenaustauscherpapieren. *Arch. Pharm.*, 296 (1963) 721–725 — R_F values of 12 vitamins.

MENKE, K. H.: Separation and identification of vitamin B_{12} analogs in the intestine of chicks. *Vitamin B_{12} intrinsic factor, 2. Europ. Symp. Hamburg*, (1961) 74–82; *C.A.*, 58 (1963) 9448b.

TAPFER, D.: (Determination of ascorbic acid in vegetable crude drugs with high ascorbic acid contents by paper chromatography). *Gyogyszereszet*, 6, No. 8 (1962) 295–300; *C.A.*, 58 (1963) 8851c.

28. ANTIBIOTICS

GALESLOOT, T. E. AND HASSING, F.: Eine schnelle und empfindliche Methode zum Nachweis von Penicillin in Milch mit Hilfe des Papierscheibchenverfahrens. *Ned. Melk Zuiveltijdschr.*, 16 (1962) 89–95; from *Z. Lebensm. Untersuch. -Forsch.*, 120 (1963) 234.

LITVINENKO, S. N.: (Chemical methods of developing paper chromatograms in work with antibiotics). *Lab. Delo*, 8, No. 9 (1962) 39–40; *C.A.*, 58 (1963) 2322d.

29. INSECTICIDES AND OTHER PESTICIDES

SKRINDE, R. T., CASKEY, J. W. AND GILLESPIE, CH. K.: Detection and quantitative estimation of synthetic organic pesticides by chromatography. *J. Am. Water Works Assoc.*, 54 (1962) 1407–1423; *C.A.*, 58 (1963) 7721b.

30. SYNTHETIC AND NATURAL DYES

DIEMAIR, W., POSTEL, W. AND SENGEWALD, H.: Untersuchungen über Anthocyane, insbesondere über das Malvin. I. Mitteilung. Beitrag zur Analytik des roten Farbstoffs in Hybridenwein. *Z. Lebensm. Untersuch. -Forsch.*, 120 (1963) 173.

JENSEN, A. AND AASMUNDRUD, O.: Paper chromatographic characterization of chlorophylls. *Acta Chem. Scand.*, 17 (1963) 907–912.

KIKUCHI, M.: Paper chromatographic identification of spot f, an unknown pigment component in *Penicillium islandicum*. *Botan. Mag. (Tokyo)*, 75 (1962) 158–160; *C.A.*, 58 (1963) 3697c.

PLÁ DELFINA, J. M.: (Synthetic dyes in food and pharmaceutical industry). *Sociedad Española de Farmacotecnia, Barcelona*, 1961, 372 p. — a review with extensive tables of R_F values.

PLÁ DELFINA, J. M.: (Systematic analysis of azo dyes used for foods, cosmetics and drugs). *Galencia Acta (Madrid)*, 15 (1962) 353–382 — 35 azo dyes.

SACCHETTA, R. A. AND SIMON, G.: (Zur Untersuchung von Paprika). *Rev. Assoc. Bioquim. Arg.*, 26 (1961) 124–131; from *Z. Anal. Chem.*, 198 (1963) 425.

SADOV, F. I. AND KRICHEVSKII, G. E.: (Determination of the reactive and hydrolysed forms of Remazol dyes). *Tekstil'n. Prom.*, 22, No. 9 (1962) 29–31; *C.A.*, 58 (1963) 3525f.

SZOKOLAY, A.: Beitrag zum papierchromatographischen und spektrophotometrischen Nachweis fettlöslicher synthetischer Farbstoffe in Lebensmitteln und Cosmetica. *Z. Lebensm. Untersuch. -Forsch.*, 120 (1963) 295.

31. PLASTICS AND THEIR INTERMEDIATES

CHANG, T. J., LIEH, H. CH. AND CH'IEN, J. Y.: (Identification of polyamides by paper chromatography). *Kao Fen Tzu T'ung-Hsun*, 4, No. 2 (1960) 93–98; *C.A.*, 58 (1963) 2506e.

32. PHARMACEUTICAL APPLICATIONS

GUVEN, K. C.: (Determination of antipyrine and salipyrine by paper chromatography). *Eczacilik Bulteni*, 4 (1962) 137–140; *C.A.*, 58 (1963) 3270c.

KÜHMSTED, H. AND WAGNER, G.: Über die Anwendung der Papierchromatographie in der Arzneianalyse. *Pharmazie*, 18 (1963), Beilage Nr. 8, *Pharmazeutische Praxis*, 152–160.

NETIEN, G. AND MERY, L.: (Chromatography of saffron adulterations). *Bull. Trav. Soc. Pharm. Lyon*, 6, No. 2 (1962) 35–39; *C.A.*, 58 (1963) 7787d.

SEIDLEIN, H. J. AND POGGENDORF, A.: Quantitativer papierchromatograpischer Nachweis von N-Acetyl-*p*-aminophenol neben anderen Analgetica. *Pharmazie*, 18 (1963), Beilage Nr. 9, *Pharmazeutische Praxis*, 192–197.

ZUNIGA, R. T.: (Application of paper chromatography and ultraviolet spectrophotometry to the control of drugging horses). *Anales Fac. Quim. Farm., Univ. Chile*, 13 (1961) 98–103; *C.A.*, 58 (1963) 2633g.

33. INORGANIC SUBSTANCES

AGRINIER, H.: (Semiquantitative determination of gold in minerals, soils, and rocks by ascending paper chromatography). *Compt. Rend.*, 255 (1962) 2801–2803; *C.A.*, 58 (1963) 2837a.

ALBERTI, G., GRASSINI, G. AND DI FERRANTE, E.: (Semiquantitative microdetermination of uranium by paper chromatography and fluorescence detection; application of the rapid analysis of natural waters). *Studi Ric. Div. Geomineraria, Comit. Nazl. Ric. Nucl.*, 3 (1960) 447–454; *C.A.*, 58 (1963) 9957g.

BECKMANN, T. J. AND LEDERER, M.: (Adsorption on cellulose of Po(IV) and other heavy metal ions from solution in HCl, HBr, and chloride salts). *Rass. Intern. Elettron. Nucl.*, 7°; 5° *Congr. Nucl., Rome*, 2 (1960) 279–304; *C.A.*, 58 (1963) 1896b.

BLASIUS, E. AND KRAEMER, R.: (Investigation of the Wackenroder reaction by means of paper radiochromatography. V. The reaction of thiosulfates with strong acids). *Z. Anorg. Allgem. Chem.*, 318 (1962) 113–121; *C.A.*, 58 (1963) 2128c.

BOCK-WERTHMANN, W.: (Specifische Entwicklungsreagentien zur Identifizierung von Kationen auf Papierchromatogrammen). *Z. Anal. Chem.*, 138 (1963) 403–407.

BOCK-WERTHMANN, W.: Ein neues universelles Entwicklungsreagenz für die Papierchromatographie von Kationen. *Anal. Chim. Acta*, 28 (1963) 519–523 — tetrahydroxyquinone.

ČERNÝ, P. AND KUBÍN, J.: (Separation and detection of iridium and rhodium by capillary chromatography). *Acta Fac. Rerum Natl. Univ. Comeniannae, Chimia*, 4 (1960) 667–672; *C.A.*, 58 (1963) 7350c.

ELBEIH, I. I. M. AND GARBA, G. G.: Determination of zirconium with hafnium in zircon via paper chromatographic separation. *Chemist-Analyst*, 52 (1963) 13–14; *C.A.*, 58 (1963) 7359h.

FRELEK, Z.: (Chromatographic and histochemical studies on metallic elements in the liver). *Ann. Univ. Mariae Curie-Sklodowska, Lublin-Polonia, Sect. D*, 16 (1961) 26–42; *C.A.*, 58 (1963) 2696c.

HALPERN, A.: Separation of iodides, iodates and periodates by paper chromatography. *Nukleonika*, 6 (1961) 107–111; *C.A.*, 58 (1963) 8383g.

KUEBLER, W.: (Qualitative and quantitative determination of phosphates in detergents by means of paper chromatography). *Textil-Rundschau*, 17 (1962) 683–684; *C.A.*, 58 (1963) 8147f.

NASCUTIU, T.: (Inorganic analysis by radioactivation of paper chromatograms). *Acad. Rep. Populare Romine, Studii Cercetari Chim.*, 10 (1962) 275–284; *C.A.*, 58 (1963) 2841g.

P'ANG, S. W. AND LIANG, S. CH.: (Separation of rare earth elements by paper chromatography). *K'o Hsueh T'ung Pao*, No. 11 (1962) 46; *C.A.*, 58 (1963) 8394c.

RIPAN, R. AND MARCU, G.: (Structure of inorganic compounds determined by radioactive isotopes. IX. The behavior of sodium tungstate with organic acids by means of radiochromatography and electrochromatography). *Studia Univ. Babes-Bolyai, Ser. 1*, No. 2 (1961) 37–43; *C.A.*, 58 (1963) 9871f.

SIKORSKA-TOMICKA, H.: (Chromatographic separation of bismuth, lead, and mercury ions). *Roczniki Akad. Med. Bialymstoku*, 7 (1962) 267–272; *C.A.*, 58 (1963) 9609d — paper impregnated with KI, caprolactam and Na hypophosphite.

TOCKSTEINOVA, D.: (Paper chromatography in the toxicologic detection of heavy metals). *Vet. Casopis*, 11 (1962) 537–540; *C.A.*, 58 (1963) 9545e.

34. RADIOACTIVE COMPOUNDS

BAILEY, R. A.: Paper chromatographic and electromigration techniques in radiochemistry. *U.S. Dept. of Comm., Office of Tech. Serv., Washington, D.C.*, 1962; *C.A.*, 58 (1963) 9636a.

MOSES, V. AND LONBERG-HOLM, K. K.: A semiautomatic device for measuring radioactivity on two-dimensional paper chromatograms. *Anal. Biochem.*, 5 (1963) 11–27.

PINTER, K. G., HAMILTON, J. G. AND MILLER, O. N.: Liquid scintillation counting with glass fiber paper. *Anal. Biochem.*, 5 (1963) 458–463.

SCHULTE, K. E. AND HENKE, G.: Der Nachweis von Radioisotopen im Harn. II. *Pharmazie*, 18 (1963) 601–616 — 36 cations.

THORSELL, W. AND NORDBERG, B. K.: $^{14}CO_2$-Fixation by *Bacillus anthracis*. *Acta Chem. Scand.*, 17 (1963) 1330–1334.

ZAKHAROVA, N. I.: (Early entrance of C_{14} into the phosphatides during photosynthesis taking place in the leaves of *Primula obsonica*). *Doklady Akad. Nauk SSSR*, 149 (1963) 202–204.

Thin-layer Chromatography

3. TECHNIQUES I

MALINS, D. C. AND WEKELL, J. C.: TLC–versatile tool. *J. Chem. Educ.*, 40 (1963) 531–534.
NAFF, M. B. AND NAFF, ANNA, S.: TLC on microscope slides. *J. Chem. Educ.*, 40 (1963) 534–535.
PITRA, J. AND ŠTĚRBA, J.: (Classification of silica gel for chromatography). *Chem. Listy*, 57 (1963) 389–391.

9. OXO COMPOUNDS

NANO, G. M. AND SANCIN, P.: Thin layer chromatography of 2,4-dinitrophenylhydrazones of aliphatic carbonyl compounds. *Experientia*, 19 (1963) 323.

10. CARBOHYDRATES

WHEELER, M., HANKE, P. AND WEILL, C. E.: Unusual carbohydrate transfer reaction of cyclodextrin transglucosylase. *Arch. Biochem. Biophys.*, 102 (1963) 397–399.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

PRIVETT, O. S. AND BLANK, M. L.: A method for the structural analysis of triglycerides and lecithins. *J. Am. Oil Chemists' Soc.*, 40 (1963) 70–75; *C.A.*, 58 (1963) 8151 b.
PURDY, S. J. AND TRUTER, E. V.: Constitution of the surface lipid from the leaves of *Brassica oleracea* (var. *capitata* (Winnigstadt)). I. Isolation and quantitative fractionation. *Proc. Roy. Soc. (London), Ser. B*, 158 (1963) 536–543.
PURDY, S. J. AND TRUTER, E. V.: Constitution of the surface lipid from the leaves of *Brassica oleracea* (var. *capitata* (Winnigstadt)). II. Esters, free primary alcohols and free acids. *Proc. Roy. Soc. (London), Ser. B*, 158 (1963) 544–552 — silica gel G impregnated with *n*-decane.
PURDY, S. J. AND TRUTER, E. V.: Constitution of the surface lipid from the leaves of *Brassica oleracea* (var. *capitata* (Winnigstadt)). III. Nonacosane and its derivatives. *Proc. Roy. Soc. (London), Ser. B*, 158 (1963) 553–565 — GLC of hydrocarbons and TLC of hydrocarbon oxidation products.

13. STEROIDS

BEŠTOVÁ, J.: (Quantitative analysis of a 7-dehydrocholesterol ester and other steroids using thin-layer chromatography on alumina). *Chem. Zvesti*, 17 (1963) 672–675.
KRITCHEVSKY, D., MARTAK, D. S. AND ROTHBLAT, G. H.: Detection of bile acids in thin-layer chromatography. *Anal. Biochem.*, 5 (1963) 388–392 — spray reagent consisting of anisaldehyde, acetic acid and sulphuric acid.
REISERT, P. M. AND SCHUMACHER, D.: Zur Trennung der C_{19}-Steroide des Harns im Dünnschichtchromatogramm. *Experientia*, 19 (1963) 84–86.
WAGENER, H. AND FROSCH, B.: Zweidimensionale dünnschichtchromatographische Trennung von Gallensäuren. *Klin. Wochschr.*, 41 (1963) 1094–1095.

14. STEROID GLYCOSIDES

HERRMANN, I. AND REPKE, K.: Über das Glukuronsäurekonjugat eines Cardenolidgenins (3-Epidigitoxigenin). *Experientia*, 19 (1963) 472–474.

18. AMINO ACIDS

PALMORK, K. H.: The use of 2,4-dinitro-fluorobenzene in the separation and identification of amino acids from sea water. *Acta Chem. Scand.*, 17 (1963) 1456–1457.
PATAKI, G. AND KELLER, M.: Dünnschichtchromatographische Bestimmung der Aminosäuren im Blut. I. Qualitative Analyse. *Z. Klin. Chem.*, 1 (1963) 157–158 — DNP amino acids.
WALZ, D., FAHMY, A. R., PATAKI, G., NIEDERWIESER, A. AND BRENNER, M.: Dünnschichtchromatographischer Nachweis der Aminosäuren im Urin. *Experientia*, 19 (1963) 213–217 — 37 amino acids in the form of DNP-derivatives.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

SCHEIG, R. L., ANNUNZIATA, R. AND PESCH, LER. A.: Studies on the separation of pyridine nucleotides by thin-layer chromatography. *Anal. Biochem.*, 5 (1963) 291–294.
WEIMANN, G. AND RANDERATH, K.: Thin-layer chromatography of deoxy-ribo-oligonucleotides. *Experientia*, 19 (1963) 49–51.

24. ORGANIC SULPHUR COMPOUNDS

KARPITSCHKA, N.: (Thin-layer chromatographic determination of sulfonamides). *Mikrochim. Ichnoanal. Acta*, (1963) 157–164; *C.A.*, 58 (1963) 10042c — R_F values of 26 sulphonamides.

25. ORGANIC PHOSPHORUS COMPOUNDS

DOIZAKI, W. M. AND ZIEVE, L.: Quantitative estimation of some phosphatides and their hydrolysis products by thin-layer chromatography. *Proc. Soc. Exptl. Biol. Med.*, 113 (1963) 91–94.

MCKILLICAN, M. E. AND SIMS, R. P. A.: Lipid changes in maturing oil-bearing plants. III. Changes in lipid classes in flax and safflower oils. *J. Am. Oil Chemists' Soc.*, 40 (1963) 108–113; *C.A.*, 58 (1963) 10414h.

PHILLIPS, B. M. AND ROBINSON, N.: Quantitative thin layer chromatography of cerebrospinal fluid phospholipids. *Clin. Chim. Acta.*, 8 (1963) 832–842.

27. VITAMINS

DILLEY, R. A. AND CRANE, F. L.: A specific assay for tocopherols in plant tissue. *Anal. Biochem.*, 5 (1963) 531–541 — comparison of thin-layer with paper chromatography.

30. SYNTHETIC AND NATURAL DYES

DRUDING, L. F.: TLC separation of ink pigments. *J. Chem. Educ.*, 40 (1963) 536.

33. INORGANIC SUBSTANCES

MERKUS, F. W. H. M.: (Use of thin-layer chromatography in toxicological analysis of metals). *Pharm. Weekbl.*, 98 (1963) 947–957 — cellulose layers.

Gas Chromatography

1. REVIEWS AND BOOKS

DIJKSTRA, A.: (Detection in gas chromatography). *Chem. Weekbl.*, 59 (1963) 429–437 — a review.

FOWLER, L.: *Gas chromatography*, Academic Press, New York, N.Y., 1963, 275 pp. — Proceedings of the Fourth International Symposium, Analysis Instrumentation Division, Instrumental Society of America, June, 1963.

HUBER, J. F. K.: Interpretation of chromatograms. *Chem. Weekbl.*, 59 (1963) 445–451 — a review of theory and practice of chromatogram evaluation.

HUYTEN, F. H.: (Application of gas chromatography in hydrocarbon chemistry). *Chem. Weekbl.*, 59 (1963) 457–464.

KARMEN, A.: Biological implications of gas chromatography. *Science*, 142 (1963) 163–172.

KNAPMAN, C. E. H.: Gas chromatography. *Nature*, 200 (1963) 639–641 — reviews of papers read at an informal symposium of the Gas Chromatography Discussion Group, Exeter University, September 27.

KNAPMAN, C. E. H. AND AMBROSE, D.: *Gas Chromatography Abstracts 1962*, Butterworths, London, 1963, 195 pp. — reviews of 763 papers published in the field of GC in 1961 and 1962.

LITTLEWOOD, A. B.: Informal symposium of the Gas Chromatography Discussion Group. *J. Gas Chromatog.*, 1, No. 11 (1963) 34–37 — see KNAPMAN, C. E. H., in *Nature*, 200 (1963) 639–641.

RIJNDERS, G. W. A.: (Principles of partition gas chromatography). *Chem. Weekbl.*, 59 (1963) 413–425 — a detailed review of the theory of the partition process in GC.

ROSSET, R.: Chromatography. *Instr. Lab.*, 25 (1963) 17–32 — review including GC apparatuses.

SHEMYAKIN, F. M.: (Chromatographic analysis of organic compounds). *Trudy Komis. Anal. Khim., Akad. Nauk SSSR.*, 13 (1963) 196–205 — an introduction speech to the chromatographic section of the analytical conference held on January 10–14, 1961, in Moscow.

VERDIN, A.: Principles and application of process gas chromatographs. *Trans. Inst. Chem. Engrs (London)*, 41, No. 3 (1963) 111–118.

2. FUNDAMENTALS, THEORY AND GENERAL

2b. Gas–solid systems

BARBER, R. M.: Two-phase flow of adsorbed vapors. *Can. J. Chem.*, 41 (1963) 1768–1781 — theory important for separation of mixtures in case of flow through microporous medium.

2c. Thermodynamics and theoretical relationships

BURROWS, G. AND PREECE, F. H.: Solubilities of vapours in relatively involatile liquids. *J. Appl. Chem. (London)*, 13 (1963) 430–443 — a theory is developed and a temperature–entropy diagram is applied to present the desorbing process.

GIDDINGS, J. C.: Kinetic origin of tailing in chromatography. *Anal. Chem.*, 35 (1963) 1999–2002 — non-linear tailing will decrease as the sample size is lowered, whereas kinetic tailing will be little affected by such changes.

GOLAY, M. J. E.: Effect of the compressibility of the carrier gas on the experimentally determined number of plates of a chromatographic column. *Nature*, 200 (1963) 776 — n^+ is unity when pressure drop $p_1 - p_0$ is small compared to p_0 and tends to $^8/_9$ as p_1 increases indefinitely.

KNYAZEV, D. A.: (Chromatographic method for measuring the separation factors of closely related substances). *Zh. Fiz. Khim.*, 37 (1963) 1190–1193.

KRIGE, G. J. AND PRETORIUS, V.: Contribution of interfacial resistance to band broadening in chromatography. *Anal. Chem.*, 35 (1963) 2009–2015 — effect of interfacial resistance proves to be negligible for chromatographic techniques with the exception of liquid–liquid chromatography.

LITTLEWOOD, A. B.: The classification of stationary liquids used in gas chromatography. *J. Gas Chromatog.*, 1, No. 11 (1963) 16–29 — specific retention of an alkane, the relative retention of successive homologues and a measure of the ability of the solvent to form hydrogen bonds are 3 definitive parameters that are useful enough to enable one to predict specific retentions.

LIU TA-CHUANG, HUANG EN-CHAI, CHANG LIANG-CH'Ü AND WANG FU-AN: (The temperature dependence of the retention volumes of homologues in gas–liquid chromatography. I. Calculation of the retention volumes at some other temperatures from the known retention at one temperature). *Acta Chim. Sinica*, 29 (1963) 228–234 (English summary, p. 235) — calculation from $\log V_g - \log V_g^\circ = m(1/T - 1/T_0)$, where $\log V_g^\circ$ and $1/T_0$ are constants depending on the nature of the functional group of the family, m a constant depending on the chromatographic system but not on the temperature.

MARTIN, H. F., DRISCOLL, J. L. AND GUDZINOWICZ, B. J.: A method for calculating gas chromatographic relative retention values for high boiling phenothiazine derivatives. *Anal. Chem.*, 35 (1963) 1901–1904 — from log t-b.p. plot on SE-30, boiling points of unknown substances were calculated and experimentally examined.

MATYSKA, B. AND DUŠKOVÁ, L.: Chromatographische Untersuchung der Eigenschaften des ternären Systems Isopren–Trimethyläthylen–polares Lösungsmittel. *Collection Czech. Chem. Commun.*, 28 (1963) 3019–3029 — retention volumes of isoprene on dimethyl sulphoxide and DEG are influenced by trimethylethylene and by concentration.

PLESSIS, L. A. DU: The quantitative treatment of plateau chromatograms obtained with complex-forming fixed phases. *J. Gas Chromatog.*, 1, No. 11 (1963) 6–9 — theoretical explanation of plateau chromatograms of ammonia on silver nitrate in benzyl cyanide.

ZHUKHOVITSKIĬ, A. A., SELENKINA, M. S., SERENKOVA, A. G. AND TURKEL'TAUB, N. M.: (Method of chromatographic identification of components of complex mixtures). *Trudy Komis. Anal. Khim., Akad. Nauk SSSR*, 13 (1963) 216–224 — linear plots $\ln V_g$ *versus* $(T_{b.p.})^2/T_{exp.}$ on silicone E-301 and $\ln V_g/(T_{exp.})^3$ *versus* $(T_{b.p.})^3/T_{exp.}$ on tricresyl phosphate for alcohols were verified.

2d. General

FOLMER, Jr., O. F.: Punched card literature retrieval system for gas chromatography. *J. Chem. Documentation*, 3 (1963) 154–156 — a critical study of optimum system handling.

LIPSKY, S. R. AND SHANIN, M. M.: Use of xenon and krypton as carrier gases for a highly sensitive detection system for gas chromatography. *Nature*, 200 (1963) 566–567 — the response to various polyatomic gases was two or three times greater than in argon.

MALINOWSKA, K.: (Comparison of plotting calibration curves on an example of separation of pyridine bases by gas chromatography). *Chem. Anal. (Warsaw)*, 8 (1963) 891–896 — calibration curves for the given compounds plotted by different methods are not always the same and give positive as well as negative responses.

3. TECHNIQUES I

3a. Detectors

BARNEY, II, J. E., STANLEY, C. W. AND COOK, C. E.: Behaviour of Demeton in electron affinity detectors. *Anal. Chem.*, 35 (1963) 2206–2208 — an anomalous response in different electron affinity detectors was found.

BERTON, A.: Chromatographie gazeuse et détection galvanique sélective. *Chim. Anal. (Paris)*, 45 (1963) 585–595 — galvanic current between two Pt-electrodes is produced by the reaction of GC fractions run to one electrode with a gauze-jacket immersed into the electrolyte.

BRESLER, S. E. AND POPOV, A. G.: (Gas–liquid chromatography. Part 3. A simple high-sensitivity ionisation detector with a corona discharge as excitation source). *Zh. Fiz. Khim.*, 37 (1963) 1178–1182.

BRUNNEE, C., JENCKEL, L. AND KRONENBERGER, K.: Fortschritte in der Anwendung von Massenspektrometer als spezifisch anzeigende Ionizationsdetektoren für die Gaschromatographie. *Z. Anal. Chem.*, 197 (1963) 42–50 — mass spectrometer with 2 ionization chambers: 20 eV and 70 eV for paraffins and aromatics.

COX, B. C.: A simple control unit for Lovelock ionisation detectors. *J. Chromatog.*, 12 (1963) 416–418 — complete electrical scheme.

IDDINGS, F. A. AND WADE, J. T.: A system for combination of gas chromatography with liquid scintillation counting. *J. Gas Chromatog.*, 1, No. 11 (1963) 31–32 — detection sensitivity better than 15 $\mu\mu$C per peak.

LEVIT, A. M.: (Calibration of chromatographic apparatus with thermochemical detectors). *Zavodsk. Lab.*, 29 (1963) 1497–1499 — thermal conductivity measurement on filament acting as catalyst of combustion.

OSTER, H.: Der Flammenionisationsdetektor für den empfindlichen Nachweis von Kohlenwasserstoffen. *Siemens-Z.*, 37 (1963) 481–487 — applicable up to 400°.

PETROCELLI, J. A.: Modified thermal conductivity detector for capillary columns. *Anal. Chem.*, 35 (1963) 2220–2221 — good response with 1 μl sample (split 100:1) in 200 ft. × 0.01 in. I.D. squalane-coated columns; no reference to the same problem treated by SASAKI, N. *et al.*: *Nature*, 186 (1963) 309–310.

SHARPLESS, N. E.: Structural effects on quantitative gas-chromatographic detectors response. Methyl esters of dicarboxylic acids. *J. Chromatog.*, 12 (1963) 401–403 — molar response in argon detection varies with carbon number; a minimum is shown in the case of succinic acid.

SHOEMAKE, G. R., LOVELOCK, J. E. AND ZLATKIS, A.: The effect of temperature and carrier gas on the loss rate of tritium from radioactive foils. *J. Chromatog.*, 12 (1963) 314–320 — maximum operating temperature for tritium foils is around 225°; with hydrogen as carrier gas leakage losses become apparent at a much lower temperature.

THOMPSON, W. R.: Reducing thermal upsets in fluid measuring cells. *U.S. Pat.* 3,097,520 (16th July, 1963).

3b. Column performance and filling studies

BAUM, E. M.: Evaluation of microporous polyethylene as a low temperature gas chromatographic support. *J. Gas Chromatog.*, 1, No. 11 (1963) 13–15 — polyethylene Mipor 14 PN-G (ESB-Reeves Corp., Glenside, Pa., U.S.A.) was examined; pore size approx. 10 μ; max. temperature 170°.

HALÁSZ, I.: Gas-solid and gas adsorption layer chromatography. *Informal GC-Symposium of Gas Chromatography Discussion Group, Exeter University, September 27, 1963* (see *Nature*, 200 (1963) 639) — reviews of the author's original work in Frankfurt.

IKEDA, R. M., SIMMONS, D. E. AND GROSSMANN, J. D.: Removal of alcohols from complex mixtures during gas chromatography. *The 145th Am. Chem. Soc. Meeting, Sept. 8–13, 1963, New York, N.Y., Div. of Agricultural and Food Chemistry*, Abstr. No. 25, p. 12A — small quantity of boric acid at the beginning of the column removed alcohols with the exception of linalool.

KIRKLAND, J. J.: Fluorine-containing polymers as solid support in gas chromatography. *Anal. Chem.*, 35 (1963) 2003–2009 — optimal coating conditions and HETP for Kel-F, Fluoropak 80 and Teflon-6 are given in connection with Chromosorb W.

MORRISSETTE, R. A. AND LINK, W. E.: Gas–liquid chromatography of polar fatty derivatives. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 24 — Versamid 900 (a polyamide) gives no tailing for polar substances such as fatty amides, other fatty derivatives etc.

PHILLIPS, C. S. G. AND SCOTT, C. G.: Modified aluminas — a progress report. *Informal GC-Symposium of the Gas Chromatography Discussion Group, Exeter University, September 27, 1963* (see *Nature*, 200 (1963) 639–640) — CuCl and $AgNO_3$ modified aluminas retard alkenes and aromatics completely.

VIRUS, W.: Gefüllte Kapillarsäulen in der Gaschromatographie. *J. Chromatog.*, 12 (1963) 406–407 — column diameters 0.5–1.0 mm, length 2–3 m, support material 0.1–0.01 mm.

ZIELIŃSKI, E.: (The application of Polish molecular sieves and activated carbons in gas chromatography). *Chem. Anal. (Warsaw)*, 8 (1963) 907–910 — separation of He, Na, H_2 on 5A molecular sieve with N_2 as carrier gas at 20°.

3c. Apparatus, accessories and materials for GC

BOWEN, S. W. AND MARKS, M. L.: Chromatography. *U.S. Pat.* 3,097,517 (16th July, 1963) — temperature programming by electroconductive column heating.

DORAN, T. AND SPERRIN, A. D.: Inexpensive sampling device for gas chromatography. *Analyst*, 88 (1963) 738–739 — for 5, 10, 15 and 20 ml samples.

DRIENOVSKÝ, P. AND KYSEL, O.: (Pyrolytic chamber for chromatographic investigation of polymers). *Chem. Zvesti*, 17 (1963) 912–915 — construction details; temperature increase was followed during pyrolysis; it gives certain maxima.

FAVRE, J. A.: Chromatographic analyzer. *U.S. Pat.* 3,097,519 (16th July, 1963) — a constituent sorting unit consists of 4 selector valves, a column and tubing accessories.

HORTON, A. D., MEYER, Jr., A. S. AND BOTTS, J. L.: Adaptation of a gas chromatograph for analysis of radioactive gas samples. *Anal. Chem.*, 35 (1963) 1988–1989 — radioactivity of C_2–C_4 hydrocarbons did not exceed a few microcuries of gamma radiation.

KAROHL, J. H.: Off-line machine reduction of gas chromatographic data. *The 7th Conference on Analytical Chemistry in Nuclear Technology, Gatlinburg, Tenn., Oct. 8–10, 1963*. Abstr. No. 46 — chromatograms are stored on magnetic tape and at increased speed converted to retention time and peak integrals.

MILLER, D. O.: A high-temperature inlet manifold for coupling a gas chromatograph to the time-of-flight mass spectrometer. *Anal. Chem.*, 35 (1963) 2033–2035 — used for materials boiling from —128° to 314° with sensitivity in the p.p.m. range.

MILLER, J. T.: Quality control instrumentation. Part. II. *Instr. Pract.*, 17 (1963) 747–751 — a BASF chromatograph with CO_2 as carrier gas for distillation plant control is described.

MUNDAY, C. W. AND PRIMAVESI, G. R.: A modular gas-chromatography system for analyzing exit streams from reactors. *Analyst*, 88 (1963) 555–557 — complex sampling scheme for normal and back flushing work.

OSTER, H. AND GRIMM, E.: Ein Prozess-Chromatograph. *Siemens-Z.*, 37 (1963) 476–481 — construction and performance details.

PETIT, J., ZAITOUN, G. AND AUFROIX, L.: Appareil simple destiné à la combustion de substances organiques, en vue de dosages ultérieurs par chromatographie en phase gazeuse. (Note de laboratoire). *Bull. Soc. Chim. France*, (1963) 2077 — device for C-determination of 3 mg samples after combustion; errors less than 1 %.

SHOJI, H., YAMAMOTO, T. AND NISHIDA, K.: The measurement of air pollutants by gas chromatography. A collection method for lower hydrocarbons. *Trans. Japan Soc. Civil Engr. (Tokyo)*, 91 (1963) 25–33 — trapping device.

WALKER, J. Q.: Capillary chromatograph column with flame detector. *Oil Gas J.*, 61, No. 14 (1963) 78–80.

YEATTS, Jr., L. B. AND RAINEY, Jr., W. T.: Preparation and storage of volatile standard solution for gas chromatographic analysis. *J. Gas Chromatog.*, 1, No. 11 (1963) 30 — a 25 ml graduate with a Teflon stopcock plug and silicone rubber septum.

ZAHUTA, J.: Use of a spinal needle as an injection device for solid substances in gas chromatography. *J. Chromatog.*, 12 (1963) 404–405 — injection needle with stilette acting as plunger (the same accessory was described in *Zavodsk. Lab.*, 28 (1962) 1506).

4. TECHNIQUES II

4a. Preparative-scale GC

KAZAKEVICH, V. E., BRAZHNIKOV, V. V., VOLKOV, S. A. AND SAKODYNSKIĬ, K. I.: (Automatic trapping in preparative chromatography). *Khim. Tekhnol. Topliv i Masel*, No. 11 (1963) 49–52 — complete electronic scheme.

PICHLER, H., SCHULZ, H., FIRNHABER, B. AND RAO, B. R.: Zerlegung destillativ schwer trennbarer Stoffgemische durch kontinuierliche Trennung unter den Bedingungen der Elutions-Gaschromatographie. *Erdöl Kohle*, 16 (1963) 577–582 — hypersorption principle connected with column filling moving along the column and countercurrent stream of carrier gas.

TAKEDA, I. AND MASIKO, Y.: Design and construction of a preparative scale gas chromatograph. *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 446–450 — 2 g sample on 600 cm × 16 mm and 480 mm × 25 mm columns.

WRIGHT, J. L.: Advanced column design for large scale gas chromatography. *J. Gas Chromatog.*, 1 No. 11 (1963) 10–12 — causes of efficiency loss in preparative scale columns are discussed and a construction of a radial-finned column with an efficiency 400–500 % higher than a conventional 1-in. I.D. column.

4c. High-speed GC

CHÉNEAU, M. AND GUIOCHON, G.: Analyse rapide par chromatographie en phase gazeuse. III. Séparation et dosage d'anésthésiques en moins de cinq secondes. *Bull. Soc. Chim. France*, (1963) 2601–2606 — ether and some chlorohydrocarbons in 2–20 sec on classical columns (I.D. 4 mm) and thermal conductivity detector.

FARRÉ-RIUS, F. AND GUIOCHON, G.: Fast analysis of gases by gas–solid chromatography. *J. Gas Chromatog.*, 1, No. 11 (1963) 33 — 315–400 μ powder of molecular sieve 5 A separates O_2, N_2, CH_4 and CO in 25 sec at 100°.

KOONS, P. D. AND WALKER, J. Q.: Faster chromatographic analyses. *Petrol. Refiner*, 42, No. 4 (1963) 133–135.

LANDAULT, C. AND GUIOCHON, G.: Analyse rapide par chromatographie en phase gazeuse. Progrès nouveaux dans la séparation des hydrocarbures légers. *Bull. Soc. Chim. France*, (1963) 2433–2437 — capillaries with alumina; from methane to butadiene in 21–150 sec.

4d. Special microtechniques

CANO, G.: Thermogravimétrie et analyse de gas simultanée. Application à l'étude de la décomposition de l'oxalate de Ca. *Bull. Soc. Chim. France*, (1963) 2540–2542 — the gases evolved were analysed by GC.

DHONT, J. H.: Identification of organic compounds from food odours by pyrolysis. *Nature*, 200 (1963) 882 — 50 μg–5 mg of vapour (C_5 -ol, -al, -one, -thiol) is injected in the presence of air into a pyrolysis tube heated to 600°. Kováts indexing.

OKAMOTO, T. AND ONAKA, T.: Reaction gas-chromatography: determination of skeletal structure by means of reaction gas chromatography. *Chem. Pharm. Bull. (Tokyo)*, 11 (1963) 1086.

OYAMA, V. I.: Use of gas chromatography for the detection of life on Mars. *Nature*, 200 (1963) 1058–1059 — detection by means of pyrolysis–GC technique; artificial examples of chromatograms of soil microorganisms given.

4e. Automation

SIEGLER, M. AND SNYDER, D. T.: A programmed analyzer with direct digital readout for measuring dissolved gases in out-of-pile corrosion facilities. *The 7th Conference on Analytical Chemistry in Nuclear Technology, Gatlinburg, Tenn., Oct. 8–10, 1963*, Abstr. No. 48 — routine determination of H_2 ($5 \cdot 10^{-4}$–10 p.p.m.), O_2 (0.05–1000 p.p.m.) and N_2 (0.06–1000 p.p.m.), non-routine determination of CH_4, CO and CO_2.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

BELLAR, T. A., BROWN, M. F. AND SIGSBY, JR., J. E.: Determination of atmospheric pollutants in the part-per-billion range by gas chromatography. *Anal. Chem.*, 35 (1963) 1924–1927 — up to 0.1 p.p.b. by concentration from 100 cm^3 gas samples.

CVETANOVIC, R. J., DUNCAN, F. J. AND FALCONER, W. E.: Gas chromatographic separation of deuterium-substituted olefins by unstable complex formation. *Can. J. Chem.*, 41 (1963) 2095–2097 — retention data of 9 olefins on $AgNO_3$-saturated ethylene glycol at —16°.

LULOVA, N. I., TARASOV, A. I., KUDRYAVCEVA, I. A. AND ZEMSKOVA, E. I.: (Analysis of gases from crude oil processing by means of chromatography). *Tr. Komis. Anal. Khim., Akad. Nauk SSSR*, 13 (1963) 238–246 — application of automatic CO_2-absorption chromatograph CHL-2 and thermal conductivity cell chromatograph CHL-3.

5b. Other hydrocarbon types

ALBERT, D. K.: Determination of C_5 to C_{11} *n*-paraffins and hydrocarbon types in gasoline by gas chromatography. *Anal. Chem.*, 35 (1963) 1918–1921 — combination of molecular sieve subtracting and GLC on N,N-bis(2-cyanoethyl) formamide was used.

ARAKI, T.: Effects of stationary liquids on the separation of the isomers of aromatic hydrocarbons in gas chromatography. *Bull. Chem. Soc. Japan*, 36 (1963) 879–884.

ARNOLD, L. K. AND LAROSE, JR., J. A.: Destructive distillation of carbonaceous Iowa shales. *Econ. Geol.*, 58 (1963) 212–217 — C_5 hydrocarbons from liquid products on Apiezon L at 150°.

BLACK, L. T. AND MUSTAKAS, G. S.: Gas chromatographic determination of residual hexane in hexane-extracted oilseed flakes. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 40 — after extraction with iso-octane in solution obtained.

BLASIUS, E. AND BEUSHAUSEN, J.: Gaschromatographische Trennung der Inhaltsstoffe des technischen Divinylbenzols. *Z. Anal. Chem.*, 197 (1963) 228–236 — retention data of numerous different impurities on three stationary phases at 135°.

ETTRE, L. S., CIEPLINSKI, E. W. AND KABOT, F. J.: Analysis of aromatics on capillary columns with poly(phenyl ether) type liquid phases. *J. Gas Chromatog.*, 1, No. 11 (1963) 38–40 — retention data of 14 C_6–C_9 aromatics at 65° and 13 condensed aromatics by PTGC.

FELDSTEIN, M. AND BALESTRIERI, S.: The detection and estimation of part per billion concentrations of hydrocarbons. *The 145th Am. Chem. Soc. Meeting, Sept. 8–13, 1963, New York, N.Y., Div. of Water and Waste Chemistry*, Abstr. No. 63, p. 25W — determination by liquid N_2 freeze-out system from 1-litre samples with flame ionization.

GERSHTEĬN, N. A.: (Chromatographic analysis of dehydrogenation and dehydration products). *Trudy Komis. Anal. Khim., Akad. Nauk SSSR*, 13 (1963) 231–238 — retention data for C_5 to C_7 unsaturated hydrocarbons on β,β'-oxydipropionitril at 4°.

HIVELY, R. A.: Assignment of *cis-trans* configuration to mono-olefin pairs by gas chromatography. *Anal. Chem.*, 35 (1963) 1921–1924 — retention data for 14 pairs of C_4–C_8 mono-olefins on 8 stationary phases at 27°, 35° and 49°.

HOFFMANN, D. AND WYNDER, E. L.: Studies on gasoline engine exhaust. *J. Air Pollution Control Assoc.*, 13 (1963) 322–327 — on DC-710 silicone fluid at 200°.

HUYTEN, I. F. H.: (Gas chromatography of hydrocarbons obtained from coal). *Chem. Weekbl.*, 59 (1963) 457–464.

INTERNATIONAL CONFERENCE OF BENZOLE PRODUCERS: The standardization of gas–liquid chromatography for the analysis of simple hydrocarbon mixtures. *J. Chromatog.*, 12 (1963) 293–304 — statistical accuracy, repeatability and reproducibility examined in 9 European laboratories for 98% benzene and 90% toluene.

IVANOVA, M. P., SIDOROV, P. I., KOSICYNA, E. I. AND GOLOVANOVA, N. I.: (Study of the composition of a gasoline fraction of Markovsk's crude oil source). *Khim. Tekhnol. Topliv i Masel*, No. 12 (1963) 13–17 — aromatics on PEGA at 145°.

KOCHLOEFL, K., SCHNEIDER, P., ŘEŘICHA, R. AND BAŽANT, V.: Untersuchung über die Zusammensetzung der Braunkohlenteerfraktion vom Sd.P. 220–280°C. II. Isoparaffinische und cycloparaffinische Kohlenwasserstoffe. *Collection Czech. Chem. Commun.*, 28 (1963) 3362–3381 — retention data of 32 steric isomers of 10 alkyldecahydronaphthalenes on Apiezon L.

KOROL', A. N.: (Gas–liquid chromatography of alkanes C_5–C_5). *Neftekhimiya*, 3 (1963) 635–641 — relative retention and activity coefficients of some hydrocarbons on non-polar stationary phase.

LULOVA, N. I., TARASOV, A. I., FEDOSOVA, A. K., LEONT'EVA, S. A. AND KVASOVA, V. A.: (Analysis of wide boiling fraction of straight run gasoline). *Khim. Tekhnol. Topliv i Masel*, No. 12 (1963) 21–28 — selectivity coefficients of hydrocarbons on 6 stationary phases.

MARTIN, R. L. AND WINTERS, J. C.: Determination of hydrocarbons in crude oil by capillary-column gas chromatography. *Anal. Chem.*, 35 (1963) 1930–1933 — retention data of 36 benzenes (up to C_{10}) and 40 C_3–C_9 paraffins and cycloparaffins.

PEPPER, J. M. AND ROBINSON, B. P.: Acid-catalysed reactions of vinyl compounds. Part. II. Products of the reaction of vinyl acetate and benzene. *Can. J. Chem.*, 41 (1963) 2103–2106 — retention data on silicone grease and Apiezon L at 230°.

SCHNEIDER, P., KOCHLOEFL, K. AND BAŽANT, V.: Untersuchung über die Zusammensetzung der Braunkohlenteerfraktion vom Sd.P. 220–280°C. III. Aromatische Kohlenwasserstoffe. *Collection Czech. Chem. Commun.*, 28 (1963) 3382–3391 — retention data for 29 alkyl naphthalenes and related compounds on Apiezon L at 150°.

SUATONI, J. C.: Determination of normal alpha-olefins by hydrobromination. *Anal. Chem.*, 35 (1963) 2196–2198 — hydrobromination of C_{12}–C_{18} olefin mixture in the presence of lauryl peroxide converts *n*-α-olefins quantitatively (± 1.3% rel.) to primary bromides which are separated selectively by 5 A molecular sieve.

VIDERGAUZ, M. S. AND AFANAS'EV, M. I.: (Chromatographic analysis of C_9–C_{12}-hydrocarbons of pyrolytic pitch). *Khim. Tekhnol. Topliv i Masel*, No. 12 (1963) 28–35 — retention ratios of aromatic hydrocarbons on E-301 at 130°.

WALKER, J. Q. AND AHLBERG, D. L.: Quantitative analysis of aromatic hydrocarbons by capillary gas chromatography. *Anal. Chem.*, 35 (1963) 2022–2027 — *m*-bis(*m*-phenoxyphenoxy)benzene used as stationary phase for 28 aromatics resolved in 17 min; retention data for 31 aromatics at 105° and 130°.

WALKER, J. Q. AND AHLBERG, D. L.: Qualitative analysis of naphthalenes by capillary gas chromatography. *Anal. Chem.*, 35 (1963) 2028–2030 — retention data of 15 naphthalenes on 3 stationary phases at 156°, 165° and 175°.

5c. Halogen derivatives of hydrocarbons

KANAZAWA, J., ONDA, K. AND SATO, R.: Rapid analysis of technical hexachlorocyclohexane by gas–liquid chromatography. *Japan Analyst*, 12 (1963) 761–762.

MARTIN, H. AND ABRAHAM, K.: Die Analyse hochfluorierter Kohlenwasserstoffe, demonstriert am Beispiel fluorierter *n*-Hexane. *Z. Anal. Chem.*, 197 (1963) 221–228 — on dinonyl phthalate.

MOGER, G. G. AND SERGEEV, G. B.: (Use of gas chromatography for the analysis of products of low-temperature olefin bromination and hydrobromination). *Vestnik Mosk. Univ., Ser. II, Khim.*, No. 2 (1963) 14–16 — retention volumes of 12 brominated C_2–C_4 hydrocarbons on tricresyl phosphate at 140°.

PICHLER, A., LEVY, G., DELUZARCHE, A. AND MAILLARD, A.: Déterminations qualitatives et quantitatives en chromatographie en phase gazeuse. Application à quelques dérivés chlorés et nitrés du butane. *Bull. Soc. Chim. France*, (1963) 1802–1805 — retention ratios and Kováts indexes for 8 chloro- and dichlorobutanes on diisodecyl phthalate and PEG 1500 at 100°.

VAN DORT, H. M. AND HAVINGA, E.: Investigations into the conformations of non-aromatic ring compounds. VI. *Cis-* and *trans-*1,3-dihalogenocyclohexanes. The reactions of 3- and 4-chloro(bromo)cyclohexane with HBr or HCl. *Proc. Roy. Dutch Acad. Sci., Ser. B, Phys. Sci.*, 66, No. 2 (1963) 45–54 — isolation on polyglycol glutarate.

VIRIN, L. I., BUSHINSKAYA, L. M. AND DZHAGACPANYAN, R. V.: (Analysis of allyl chloride by means of gas–liquid chromatography). *Zavodsk. Lab.*, 29 (1963) 1301–1302 — on tricresyl phosphate at 70°; errors 5.5 % rel.

YAROSLAVSKAYA, S. F.: (Determination of components of some volatile mixtures in the atmosphere of industrial plants by means of gas–liquid partition chromatography). *Trudy Komis. Anal. Khim., Akad. Nauk SSSR*, 13 (1963) 269–276 — details of some trapping devices.

7. PHENOLS

BAER, H., SRINIVASAN, S., BOWSER, R. T. AND KARMEN, A.: The quantitative assay of the active principles of poison ivy by biological and gas chromatographic method. *J. Allergy*, 34 (1963) 221–224 — acetylated pentadecylcatechols on SE-30 and neopentyl GS at 205°.

KOLŠEK, J. AND MATIČIČ, M.: Beitrag zur gaschromatographischen Bestimmung einwertiger Phenole aus dem Schwelteer. *J. Chromatog.*, 12 (1963) 305–313 — retention data for 13 C_6–C_8 phenols on 5 stationary phases at 90–130°.

9. OXO COMPOUNDS

BRANDENBURG, C. F., COOK, G. L., HAINES, W. E. AND LATHAM, D. R.: Identification of a cycloalkyl ketone in Wilmington petroleum through use of chromatography and spectroscopy. *The 145th Am. Chem. Soc. Meeting, Sept. 8–13, 1963, New York, N.Y., Division of Petroleum Chemistry*, Abstr. No. 40, p. 15 S.

COBB, W. Y., PATTONS, S. AND GRILL, H.: Occurrence of vanilin in heated milks. *J. Dairy Sci.*, 46 (1963) 566–567 — retention data on DEGA and Carbowax 1540 at 175° and 202°.

10. CARBOHYDRATES

BISHOP, C. T., COOPER, F. P. AND MURRAY, R. K.: Reactions of carbohydrate derivatives during gas–liquid chromatography. *Can. J. Chem.*, 41 (1963) 2245–2250 — 4 types of chemical changes are demonstrated.

GORIN, P. A. J. AND SPENCER, J. F. T.: Structural relationships of extracellular polysaccharides from phytopathogenic *Xanthomonas* spp. *Can. J. Chem.*, 41 (1963) 2357–2361 — di- to tetra-O-methylglucosides on fully methylated hydroxyethylcellulose WP-09 (Union Carbide) at 220°.

HORIGUCHI, M., HIGOSAKI, N. IWASHIGE, T. AND IWAI, I.: Gas partition chromatography of 2,3-anhydro-D-ribofuranoside and 2,3-anhydro-D-xylofuranoside. *Chem. Pharm. Bull. (Tokyo)*, 11 (1963) 1055–1058.

PERLIN, A. S. AND TABER, W. A.: A glucan produced by *Claviceps purpurea*. *Can. J. Chem.*, 41 (1963) 2278–2282 — di- to tetra-O-methyl methylhexanosides by PTGC 60°–200° on Craig ester.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

ACKMAN, R. G., BURGHER, R. D. AND SIPOS, J. C.: Quantitative gas–liquid chromatography of the higher fatty acids. *Nature*, 200 (1963) 777–778 — formic acid vapour acts as tailing reducer for free C_{16}–C_{18} (18:1, 18:2, 18:3) acids; temperature 212°; EGS; flame ionization.

ARNOLD, L. K. AND MILLOY, A. D.: Distribution of individual fatty acids in the crystalline fraction of lard. *J. Am. Oil Chemists' Soc.*, 40 (1963) 296–297 — C_{14}–C_{18} fatty acids on PEGS and BuGS at 215° and 220°.

BELL, E. W., FRIEDRICH, J. P., GAST, L. E. AND COWAN, J. C.: Preparation of alcohols from fatty cyclic acids. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 28 — saturated C_{18} and C_{20} cyclic alcohols.

BRENNER, R. R., VAZZA, D. V. AND DETOMAS, M. E.: Effect of a fat-free diet and of different dietary fatty acids (palmitate, oleate and linoleate) on the fatty acid composition of fresh-water fish lipids. *J. Lipid Res.*, 4 (1963) 341–345 — fatty acid esters on PEGA at 180°.

BUTTERFIELD, R. O., SCHOFFIELD, C. R. AND DUTTON, H. J.: Preparation of 9,15-octadecadienoate isomers. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 14 — identification by means of capillary GLC.

CAPELLA, P. AND JACINI, G.: (Normalisation of the gas chromatographic method; present possibilities and relative difficulties). *Riv. Ital. Sostanze Grasse*, 40 (1963) 317–320 — for fatty acid analysis; esterification methods are discussed.

CORNELINO, J. A. AND SHONE, G.: Cyclopropenoid fatty acids of *Bombax oleagineum* seed oil. *Chem. Ind. (London)*, (1963) 1246–1247 — methyl esters on PEGA at 160° and 175°.

CRAIG, B. M. AND BHATTY, M. K.: A naturally occurring all-*cis* 6,9,12,15-octadecatetraenoic acid in plant oils. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 9 — identification by GLC.

DIETZ, R. W., PICKETT, B. W., KOMAREK, R. J. AND JENSEN, R. G.: Fatty acid composition of bovine semen. *J. Dairy Sci.*, 46 (1963) 468–472 — on DEGS at 178–182°.

FARSTAD, M. AND SKAUG, O. E.: Determination of higher fatty acids in the cerebrospinal fluid by gas chromatography. *Scand. J. Clin. Lab. Invest.*, 15 (1963) 255–258 — methyl esters of C_{14}–C_{22} fatty acids on Apiezon L at 210°.

FEDELI, E., CAPELLA, P. AND VALENTINI, A.: (Estimation of double bond position of fatty acids). *Riv. Ital. Sostanze Grasse*, 40 (1963) 313–316 — hydroxylation with peracetic acid and oxidation with IO_4^- gives fragment for GC without secondary products.

FRANKEL, E. N., DAVISON, V. L., EMKEN, E. AND DUTTON, H. J.: Homogeneous catalytic hydrogenation of unsaturated fats: Cobalt carbonyl. *J. Am. Oil. Chemists' Soc.*, 40, No. 9 (1963) 33.

GOMPERTZ, S. M. AND SAMMONS, H. G.: The origin of faecal lipids. The composition of faecal fats in human subjects. *Clin. Chim. Acta*, 8 (1963) 591–603 — methyl esters of long-chain fatty acids on PEGA and Apiezon L at 197°.

GRYNBERG, H., SZCZEPAŃSKA, H. AND BEDOWICZ, M.: (The importance of the scheme of some conditions of analysis in the determination of the composition of fatty acids by gas–liquid chromatography). *Chem. Anal. (Warsaw)*, 8 (1963) 881–890 — methyl esters of C_6–C_{25} fatty acid on Apiezon L and PEGS at 150°–250°.

HUGHES, G., KELLY, V. J. AND STEWART, R. A.: Linoleic acid — an essential nutrient. Its content in infant formulas and precooked cereals. *Clin. Pediat.*, 2 (1963) 555–561 — as methyl esters on DEGS at 190°.

JAMES, A. T.: Method of separation of long-chain unsaturated fatty acids. *Analyst*, 88 (1963) 572–582.

JÜTTNER, B.: Oxydation von Steinkohle im alkalischen Medium mit Chlor und Manganoxyd. *Brennstoff-Chem.*, 44 (1963) 206–210 — methyl esters of benzene mono- to hexacarboxylic acids on silicone rubber gum by PTGC 150–310°.

KAYAMA, M., TSUCHIYA, Y., NEVENZEL, J. C., FULCO, A. AND MEAD, J. F.: Incorporation of linolenic-1-C^{14} acid into eicosapentaenoic and docosahexaenoic acids in fish. *J. Am. Oil Chemists' Soc.*, 40 (1963) 499–502 — retention of methyl esters on EGS at 196°.

LITCHFIELD, C., FARQUHAR, M. AND REISER, R.: The triglyceride composition of *Cuphea miniata* seed fat. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 14.

LITCHFIELD, C., RIESER, R. AND ISBELL, A. A.: The analysis of *cis-trans* fatty acid isomers using gas–liquid chromatography. *J. Am. Oil. Chemists' Soc.*, 40 (1963) 302–309 — retention data of stereoisomers of monosaturated, conjugated and epoxy fatty acids.

LUDDY, F. E., BARFORD, R. A., HERB, S. F. AND RIEMENSCHNEIDER, R. W.: Pancreatic lipase hydrolysis of triglycerides by a semimicro technique. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 14.

LUKE, H. H., FREEMAN, T. E. AND KIER, L. B.: Identification of the methyl esters of the stable Krebs cycle acids by gas liquid chromatography. *Anal. Chem.*, 35 (1963) 1916–1918 — retention of methyl esters of Krebs di- and tricarboxylic acids on LAC-728 and silicone grease by PTGC 90–225°.

MCCARTHY, M. J. AND KUKSIS, A.: Exploration of the selectivity of various chromatographic techniques for the study of the triglyceride structure of natural fats. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 9.

NELSON, J. H., GLASS, R. L. AND GEDDES, W. F.: The triglycerides and fatty acids of wheat. *Cereal Chem.*, 40 (1963) 343–351 — on butanediol succinate polyester at 210°.

PELICK, N., HENLY, R. S., SWEENY, R. F. AND MILLER, M.: Special methods of purifying fatty acids. *J. Am. Oil Chemists' Soc.*, 40 (1963) 419–425 — GLC and TLC for about 10 mg samples.

PRYDE, E. H., ANDERS, D. E., TEETER, H. M. AND COWAN, J. C.: Unsaturated aldehyde oils by the partial ozonization of soybean oil. *J. Am. Oil Chemists' Soc.*, 40 (1963) 497–499 — retention data of fatty acids methyl esters on PEGS at 160°.

RAHM, J. J. AND HOLMAN, R. T.: The relationship of single dietary polyunsaturated acids to the fatty acid composition of lipids from subcellular particles and unfractionated tissues. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 42.

TULLOCH, A. P.: The gas liquid chromatography of hydroxystearic acid esters. *J. Am. Oil. Chemists' Soc.*, 40, No. 9 (1963) 24 — 17 isomeric acids examined on several stationary phases.

WILLIAMS, C. M., DORIN, G. E. AND GREEN, M.: Determination of β-phenylactic acid by gas chromatography. *Anal. Biochem.*, 6 (1963) 468–475 — on EGA at 172°.

YOUNGS, C. G. AND SUBBARAM, M. R.: Determination of the glycide structure of fats: Gas liquid chromatography of oxidized glycerides. *J. Am. Oil. Chemists' Soc.*, 40, No. 9 (1963) 14.

13. STEROIDS

BAILEY, E.: The quantitative gas chromatography of some corticosteroids. *J. Endocrinol.*, 26, No. 4 (1963) 27.

KEUTMANN, E. H., SPARAGANA, M. AND MASON, W. B.: Gas chromatographic determination of individual 17-ketosteroids in urine. Studies on normal human subjects. *Clin. Chem.*, 9 (1963) 493–493.

MIKITA, M. AND WELLS, W. W.: Quantitative analysis of fecal bile acids by gas–liquid chromatography. *Anal. Biochem.*, 5 (1963) 523–430 — retention data of deoxycholic and lithocholic acids at 245° and 280°.

THOMPSON, M. J., DUPONT, J. AND ROBBINS, W. E.: The sterols of liver and carcass of 20,25 diaza-cholesterol-fed rats. *Steroids*, 2 (1963) 99–104 — retention data on QF-1 and NGS at 204° and 215°.

VANDENHEUVEL, W. J. A. AND HORNING, E. C.: Gas-chromatographic characterisation of steroid ketones as N,N-dimethylhydrazones. *Biochim. Biophys. Acta*, 74 (1963) 560–563.

WOTIZ, H. H.: Studies in steroid metabolism. XIV. Modification of the analysis of urinary estrogens by gas chromatography. *Biochim. Biophys. Acta*, 74 (1963) 122–126 — retention data on SE-30 at 240°.

15. TERPENE DERIVATIVES

HARTMANN, H., OAKS, D. AND DIMICK, K. P.: Essential oil analysis by two-channel gas chromatography. *The 145th Am. Chem. Soc. Meeting, Sept. 8–13, 1963, New York, N.Y., Division of Agricultural and Food Chemistry*, Abstr. No. 24, p. 11 A — simultaneous action of flame ionization and electron capture detector.

HELLYER, R. O. AND MCKERN, H. H. G.: The volatile oils of the genus eucalyptus (family *Myrtaceae*). II. *Australian J. Chem.*, 16 (1963) 515–519 — terpenes on silicone DC 550 at 170°.

HOWARD, G. E.: The analysis of germanium oil by gas chromatography. *Analyst*, 88 (1963) 633–642 — on PEG 400, polypropylene glycol succinate, diglycerol and PEG salicylate at 200°.

HÜCKEL, W. AND SCHEEL, J.: Änderungen des Molekülbau bei chemischen Reaktionen. XI. Die Umsetzung von *endo-* und *exo*-Fenchylamin mit salpetriger Säure. IV. Gaschromatographie und IR-Spektroskopie als analytische Verfahren in der Terpenreihe. *Ann.*, 664 (1963) 19–31 — retention data on Carbowax K 4000, silicone grease at 125°, 130° and 175°.

MITZNER, B. M., THEIMER, E. T. AND LEMBERG, S.: 1(7),4(8)-*p*-Menthadiene. *Can. J. Chem.*, 41 (1963) 2097–2099 — on Reoplex 400 at 200°.

NIGAM, I. C. AND LEVI, L.: Column and gas chromatographic analysis of oil of wild ginger. Identification and estimation of some new constituents. *Can. J. Chem.*, 41 (1963) 1726–1730.

RUDLEFF, E. VON: Gas–liquid chromatography of terpenes. Part. VIII. The volatile oil of *Tanacetum vulgare* L. *Can. J. Chem.*, 41 (1963) 1737–1743 — 27 terpenes on polyester stationary phases by TPGC 65–180°.

YAMANISHI, T., KIRIBUCHI, T., SAKAI, M., FUJITA, N., IKEDA, Y. AND SASA, K.: Studies on the flavor of green tea. Part V. Examination of the essential oil of the tea leaves by gas liquid chromatography. *Agr. Biol. Chem. (Tokyo)*, 27 (1963) 193–198 — retention data on PEG and PEGA at 150°.

22. ALKALOIDS

ARNDT, R. R., BAARSCHERS, W. H., DOUGLAS, B., SHOOP, E. C. AND WEISBACH, J. A.: Gas–liquid chromatography of aporphine alkaloids. *Chem. Ind. (London)* (1963) 1163–1165 — retention data of alkaloids and ethyl ethers of natural phenolic alkaloids on SE-30 at 235° and 250°.

24. ORGANIC SULPHUR COMPOUNDS

CATES, V. E.: A study of sulphoxides and sulphones and a method of structural analysis using gas chromatography. *Dissertation Abstr.*, 24, No. 1 (1963) 49 — best stationary phases are Carbowax 1500 and 20M.

GRIESBAUM, K., OSWALD, A. A., QUIRAM, E. R. AND NAEGELE, W.: Allene chemistry. I. Free radical addition of thiols to allene. *J. Org. Chem.*, 28 (1963) 1952–1957.

OSWALD, A. A., GRIESBAUM, K. AND HUDSON, Jr., B. E.: Organic sulphur compounds. Amine–hydroperoxide complexes as intermediates in the co-oxidation of thiols with 2,5-dimethyl-2,4-hexadiene. *J. Org. Chem.*, 28 (1963) 2351–2354.

26. METALLO-ORGANIC COMPOUNDS

BONELLI, E. J. AND HARTMANN, H.: Determination of lead alkyl by gas chromatography with electron capture detector. *Anal. Chem.*, 35 (1963) 1980–1981 — retention data on TCEP at 72° with 0.1 μl sample of 4 c.c. per gallon of gasoline.

POLLARD, F. H.: Gas chromatographic studies of compounds of group IV A elements. *Informal GC-Symposium of the Gas Chromatography Discussion Group, Exeter University, September 27, 1963*; see *Nature*, 200 (1963) 640–641 — organo-silicon, -germanium, -tin and -lead compounds on 9 stationary phases.

ROTZSCHE, H.: Silikone. Mitteilung 68. Gas-Chromatographie der Silane in Chlorsilanen. *Z. Anorg. Chem.*, 324 (1963) 197–201.

29. INSECTICIDES AND OTHER PESTICIDES

BONELLI, E., DIMICK, K. P. AND HARTMANN, H.: GC retention time and sensitivity data for insecticides and herbicides. *The 145th Am. Chem. Soc. Meeting, Sept. 8–13, 1963, New York, N.Y., Division of Agricultural and Food Chemistry*, Abstr. No. 48, p. 20A — retention data on DOW-11.

DUGAN, P. R., NAIR III, J. H., O'NEILL, R. D. AND SPRAGUE, M. L.: Pesticide analysis in a New York State watershed, an application of electron capture gas–liquid chromatography. *The 145th Am. Chem. Soc. Meeting, September 8–13, 1963, New York, N.Y., Division of Agricultural and Food Chemistry*, Abstr. No. 49, p. 20A.

HAYES, Jr., W. J. AND DALE, W. E.: Storage of insecticides in French people. *Nature*, 199 (1963) 1189–1191 — on high-vacuum silicone grease at 215°.

KANAZAWA, J.: Determination of chloropicrin in fumigants by gas liquid chromatography. *Agr. Biol. Chem. (Tokyo)*, 27 (1963) 159–161 — retention data on PEG 6000 at 100°.

KANAZAWA, J. AND SATO, R.: Determination of pentachloronitrobenzene in dust by gas–liquid chromatography. *Japan Analyst*, 12 (1963) 758–760.

ROBINSON, J. AND RICHARDSON, A.: The separation of chlorinated insecticides and related compounds. *Chem. Ind. (London)*, (1963) 1460–1462 — retention data of 8 compounds on 4 stationary phases at 160–170°.

31. PLASTICS AND THEIR INTERMEDIATES

BOMBAUCH, K. J., COOK, C. E. AND CLAMPITT, B. H.: Investigation of comonomer distribution in ethylene–acrylate copolymers with thermal methods. *Anal. Chem.*, 35 (1963) 1834–1837 — the major acrylate pyrolysate was shown to be inversely related to ethylene–acrylate junctions.

JONES, C. E. R. AND REYNOLDS, G. E. J.: Polymer pyromania. *Informal GC-Symposium of the Gas Chromatography Discussion Group, Exeter University, September 27, 1963*; see *Nature*, 200 (1963) 641 — criteria for analytical pyrolysis–GC technique were discussed.

RYBICKA, S. M.: The analysis of surface coating resins by pyrolysis-gas chromatography. *Informal GC-Symposium of the Gas Chromatography Discussion Group, Exeter University, September 27, 1963*; see *Nature*, 200 (1963) 640 — types of pyrolysis processes were discussed.

32. PHARMACEUTICAL APPLICATIONS

GALLO, G. G. AND CHIESA, L.: (Gas chromatographic analysis of solvents in pharmaceutical preparations). *Farmaco (Pavia), Ed. Prat.*, 18 (1963) 206–213 — retention data at 100°, 170° and 220°.

MACDONALD, A. AND PFLAUM, R. T.: Gas chromatographic data for some antihistamines. *J. Pharm. Sci.*, 52 (1963) 816 — retention data of 19 antihistamines on SE-30 at 173°.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

BOURKE, P. J., DAWSON, R. W. AND DENTON, W. H.: Analysis of v.p.m. impurities in helium by gas chromatography and ionization. *The 7th Conference on Analytical Chemistry in Nuclear Technology, Gatlinburg, Tenn., Oct. 8–10, 1963*, Abstr. 50 — a small purifier for He (impurities 0.1 v.p.m.) and determination of 0.02 v.p.m. CO_2 and 0.05 v.p.m. CO; decreasing sensitivity in the direction O_2, Ar, N_2 and H_2.

HACHENBERG, H. AND GUTBERLET, J.: Verbesserung der Stickstoffbestimmung nach Dumas durch die Gaschromatographie. *Brennstoff-Chem.*, 44 (1963) 235–237.

KOHLSCHÜTTER, H. W. AND HÖPPE, W.: Gas-Adsorptions-Chromatographie mit aktivem Chrom-(III)oxid. *Z. Anal. Chem.*, 197 (1963) 133–140 — Cr_2O_3 activated at 550° in H_2 gives hydrogenation of O_2 in gaseous mixtures of argon.

LAMONT, B. D., JOHNSON, J. D. AND DERBY, J. V.: Analysis of uranium carbide. *The 7th Conference on Analytical Chemistry in Nuclear Technology, Gatlinburg, Tenn., Oct. 8–10, 1963*, Abstr. No. 49 — carbide is extracted (hot vacuum technique) for H_2 and reacted with BrF_3 for O_2 and N_2.

LIKHACHEV, A. D.: (Chromatographic determination of carbon monoxide and oxygen in products of fuel combustion). *Zavodsk. Lab.*, 29 (1963) 1302–1304 — separation of H_2, N_2, CO and CH_4 on charcoal.

REIN, H. T., MILVILLE, M. E. AND FAINBERG, A. H.: Separation of oxygen and nitrogen by packed-column chromatography at room temperature. *Anal. Chem.*, 35 (1963) 1536.

33b. Volatile inorganic compounds

BIGHI, C.: (Gas-chromatographic study of technical dithiocarbamic acid). *Ann. Chim. (Rome)*, 53 (1963) 1061–1067.

BRADLEY, H. L. AND HOORE, C. R.: Application of gas chromatography to the determination of carbon in sodium. *The 7th Conference on Analytical Chemistry in Nuclear Technology, Gatlinburg, Tenn., October 8–10, 1963*, Abstr. No. 47 — Na is dissolved in ethanol–water mixture and after acidification with H_2SO_4, CO_2 is collected and determined.

HAMLIN, A. G., IVESON, G. AND PHILLIPS, T. R.: Analysis of volatile inorganic fluorides by gas liquid chromatography. *Anal. Chem.*, 35 (1963) 2037–2043 — retention data of 12 inorganic halogen derivatives, *e.g.* Cl_2, ClF_3, ClO_2, UF_6 on KEL-F at 60°; nickel katharometer; split-column system (scheme) for rapid automatic analysis.

NESTLER, H. AND BERGER, W.: Gas-chromatographische Untersuchungen von Retortenabgasen der CS_2-Produktion. *Chem. Tech. (Berlin)*, 15 (1963) 616–617 — retention data of CO_2, H_2S, COS and CS_2 on squalane; errors max. ± 2% abs.

SCHIER, K. H. AND BAUER, W.: Untersuchung über im Glas gelöste Gase mit Hilfe einer induktiv beheizten Extraktionsapparatur. *Silikattech.*, 14 (1963) 72–75 — O_2, N_2, CO_2 and SO_2 with thermal conductivity cell and H_2 as carrier gas.

SCHIER, K. H. AND BAUER, W.: Gas-chromatographische Untersuchung des Inhalts von Blasen im Glas. *Silikat Tech.*, 14 (1963) 119–121 — H_2, O_2, NO_2, CO, CO_2 and SO_2 with β-ionization detector with Ne as carrier gas.

STASZEWSKI, R., POMPOWSKI, T. AND JANÁK, J.: Analysis of the mixture CO_2, H_2S, COS, CS_2 and SO_2 by gas–liquid chromatography. *Chem. Anal. (Warsaw)*, 8 (1963) 897–905 — retention data on squalane, dinonyl phthalate and PEG 400 (porous Teflon) at 22° and 46°.

STUCKEY, W. K. AND WALKER, J. M.: A combustion–gas chromatographic method for the simultaneous determination of carbon and sulphur in ferrous metals. *Anal. Chem.*, 35 (1963) 2015–2017 — C in 0.011 to 3.28% and S in 0.011–0.329% with errors ± 2% rel. for C and ± 5% rel. for S on silica gel column after combustion in pressurized oxygen in a high-frequency induction furnace in 17 min; no reference is made to the same problem dealt with by J. JURÁNEK: *Collection Czech. Chem. Commun.*, 25 (1960) 2814–2821.

WALKER, J. M. AND KUO, C. W.: Carbon determination on ferrous metals by gas chromatography. *Anal. Chem.*, 35 (1963) 2017–2019 — CO_2 was trapped in 5A molecular sieve column and desorbed at 275°; detection limit is 0.0005%; analysis time 20 min.

34. RADIOACTIVE COMPOUNDS

CLULEY, H. J. AND KONRATH, J. H.: Analysis of ^{14}CO–$^{14}CO_2$ mixtures by gas chromatographic separation and aqueous solution counting. *Analyst*, 88 (1963) 761–770.

DUTTON, H. J. AND MOUNTS, T. L.: Micro vapor-phase hydrogenation monitored with tandem chromatography-radioactivity. *The 145th Am. Chem. Soc. Meeting, September 8–13, 1963, New York, N.Y., Division of Colloid and Surface Chemistry*, Abstr. No. 59, p. 22I — labelled fatty acid methyl esters.

GELLERMAN, J. L. AND SCHLENK, H.: The preparation of ^{14}C-labeled poly-unsaturated fatty acids from *Ochromonas damica*. *J. Am. Oil Chemists' Soc.*, 40, No. 9 (1963) 9 and 14.

KARMEN, A., WHYTE, M. AND GOODMAN, D. W. S.: Fatty acid esterification and chylomicron formation during fat absorption. I. Triglycerides and cholesterol esters. *J. Lipid Res.*, 4 (1963) 312–321 — labelled fatty acids on PEGA at 200°.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

ALTSHULLER, A. P.: Analysis of organic substances in the atmosphere by gas chromatographs with ionization detectors. *Intern. J. Air Water Pollution*, 7, No. 2/3 (1963) 87–89 — flame ionization up to 0.005 p.p.m., after concentration up to 0.1 p.p.b.

ANDERSON, R. H. AND HELSON, J. P.: A method for the determination of BHA and BHT in cereal products. *Food Technol.*, 17, No. 7 (1963) 95–96 — PTGC, 125–190°, for butylated hydroxyanisole and butylated hydroxytoluene.

BLOURI, B., FAUVET, J. É. AND RUMPF, P.: Analyse chromatographie en phase gazeuse de mélanges d'hydrocarbures aliphatiques non saturés, à longues chaînes, ou de dérivés halogénés ou hydroxy-halogénés. *Bull. Soc. Chim. France,* (1963) 1855–1860 — retention data for heptene-1,-2 and -3, octene-1,-2 and -3, and decene-1,-2,-3 and -4 on PEG 1500 at 45–90°, 2-bromobutanes and 4-bromoheptanes on PEG 400 and 1500 at 75–85°, 3-chloropentanes, 4-chloroheptanes and 5-chlorodecanes on PEG 1500 at 60–115° and two C_7 chlorohydrins on PEG 1500 at 100°.

COSGROVE, D. J. AND TATE, M. E.: Occurrence of *neo*-inositol hexaphosphate in soil. *Nature,* 200 (1963) 568–569 — separated as acetate.

EISENBRAUN, E. J., BADER, A. R., POLACHECK, J. W. AND REIF, E.: Hydrogen peroxide–vanadium pentoxide oxidation of cyclohexenes. *J. Org. Chem.,* 28 (1963) 2057–2062 — retention data of 2-cyclohexen-1-ol and -1-one on phenyldiethanolamine succinate.

GADSDEN, R. H. AND MCCORD, W. M.: A preparative technique for isolation of materials of relatively high volatility for gas-chromatographic quantitation. *Clin. Chem.,* 9 (1963) 467.

GILDENBERG, L. AND TROWBRIDGE, J. R.: Gas liquid chromatography of acetate esters of nonionic surfactants. *J. Am. Oil Chemists' Soc.,* 40, No. 9 (1963) 23.

HILL, F. D. AND HAMMOND, E. G.: The flavor of autoxidized soybean oil. *J. Am. Oil Chemists' Soc.,* 40, No. 9 (1963) 27 — aldehydes and ketones on BuGS.

HIROTA, K., IIZUKA, S., OCHI, H. AND HATADA, M.: Radiation-induced addition of acetaldehyde to ethylene. *Bull. Chem. Soc. Japan,* 36 (1963) 115–120.

HOH, G. L. K., BARLOW, D. D., CHADWICK, A. F., LAKE, D. B. AND SHEERAN, S. R.: Hydrogen peroxide oxidation of tertiary amines. *J. Am. Oil Chemists' Soc.,* 40 (1963) 268–271 — dimethyl dodecylamine and 1-dodecene on Apiezon L at 225°.

HÜCKEL, W. AND BROSS, R.: Beiträge zur Konstellationsanalyse. X. (Solvolyse von Toluolsulfonsäureestern. XIII.). *Cis-* und *trans*-3-isopropylcyclopentanol. *Ann.,* 664 (1963) 1–19 — retention data on PEG, $AgNO_3$ PEG K4000 at 38° and 100–130°.

JACOBS, E. S.: Gas chromatography of aqueous solutions. Determination of hydrocarbons and halocarbons. *Anal. Chem.,* 35 (1963) 2035–2037 — a precolumn with P_2O_5 on Desicote- or Siliclad-treated firebrick (9:1) used for over 50 analyses with 10 μl samples.

KENNEY, H. E., KOMANOWSKY, D. AND WRIGLEY, A. N.: Reaction of dichloro-carbene with fats and oils. *J. Am. Oil Chemists' Soc.,* 40, No. 9 (1963) 30.

MCKILLICAN, M. E.: Studies of the phospholipid–glycolipid complex of wheat endosperm. *J. Am. Oil Chemists' Soc.,* 40, No. 9 (1963) 40 — fatty acids and sterols.

MAHADEVAN, V., CUBERO, E. AND LUNDRERG, W. O.: Platelet phosphatides, their separation, fatty acid composition and clotting activity. *J. Am. Oil. Chemists' Soc.,* 40, No. 9 (1963) 42 — fatty acids and aldehydes as methyl esters and dimethyl acetals.

MEHRENS, J. E.: The determination of Dowtherm A in fatty acids. *J. Am. Oil Chemists' Soc.,* 40, No. 9 (1963) 26 — in mixtures with C_{10}–C_{22} fatty acids on DEGS at 180°.

MENGUY, P., CHAUVEL, A., CLÉMENT, G. AND BALACEANU, J. C.: Hydroperoxydes isomères obtenus par l'oxydation en phase liquide des butènes-1 et -2 et du méthyl-3-butène-1. *Bull. Soc. Chim. France,* (1963) 2643–2651 — preparative and analytical GC of hydrocarbons, alcohols and bromo-derivatives.

NAVES, Y. R.: Errors in application of instrumentation to the analysis of perfumery raw materials. *J. Soc. Cosmetic Chemists,* 14 (1963) 29–44 — review of GC possibilities.

NORMAN, V., NEWSONE, J. R. AND KEITH, C. H.: Vapor phase analysis of tobacco smoke. *The 145th Am. Chem. Soc. Meeting, September 8–13, 1963, New York, N.Y., Division of Agricultural and Food Chemistry,* Abstr. No. 55, p. 23A.

RAZAFINDRAZAKA, J. AND METZGER, J.: La cire de sisal de Madagascar. II. Étude quantitative des constituants par chromatographie en phase gazeuse. *Bull. Soc. Chim. France,* (1963) 1633–1637 — retention data for C_{27}–C_{33} hydrocarbons and C_{26}–C_{32} monoalcohols on silicone 710 at 310°, C_{14}–C_{24} and C_{24}–C_{34} fatty acids on DEGS at 190° and 230° and C_{22}–C_{24} hydroxy fatty acids on Apiezon M at 210°.

SELF, R. AND SWAIN, T.: Flavour in potatoes. *Proc. Nutr. Soc.,* 22 (1963) 176–182 — aldehydes, ketones and sulphur compounds.

SRINIVASAN, S., BAYER, H., BOWSER, R. AND KARMEN, A.: An assay for pentadecylcatechols in poison ivy extracts by gas chromatography. *Anal. Biochem.,* 6 (1963) 234–239 — retention data on SE-30 and neopentyl glycol succinate at 205°.

SWOBODA, P. A. T. AND LEA, C. H.: Research into fat rancidity by gas–liquid chromatography. *J. Am. Oil Chemists' Soc.,* 40, No. 9 (1963) 26 — flavour constituents.

TURKEL'TAUB, N. M., SHEMYATENKOVA, V. T., AÏNSHTEÏN, S. A. AND SYAVCILLO, S. V.: (Determination of some organic impurities in raw material and half products of synthesis of silicon organic compounds). *Trudy Komis. Anal. Khim., Akad. Nauk SSSR,* 13 (1963) 284–289 — quantitative determination of alcohol, ether and benzene.

VLODAVEC, M. L., GOL'BERT, K. A., CHERBINSKAYA, E. YA. AND NAZAROVA, N. N.: (Determination of content of carbonyl compounds and allyl alcohol which are produced by the contact reaction of acrolein with ethyl and isopropyl alcohols). *Trudy Komis. Anal. Khim., Akad. Nauk SSSR*, 13 (1963) 209–216 — retention data of 11 oxygenated compounds on β,β'-thiopropionitrile at 72°.

WHEATLEY, V. R.: Some applications of gas chromatography to cosmetology. VI. Cosmetic instrumental analysis. *Am. Perfumer Cosmet.*, 78, No. 1 (1963) 27–30, 32–33 — review of 32 GC-applications.

WONG, N. P.: A comparison of the volatile compounds of fresh and decomposed cream by gas chromatography. *J. Dairy Sci.*, 46 (1963) 571–573.

JOURNAL OF CHROMATOGRAPHY VOL. 14 (1964)

AUTHOR INDEX

JOURNAL OF CHROMATOGRAPHY VOL. 14 (1964)

SUBJECT INDEX

ERRATA

J. Chromatog., 11 (1963) 472.

"Sulfolene" and "Butadiene Sulfone" are both the same compound and are the common names for 2,5-Dihydrothiophene-1,1-dioxide.

J. Chromatog., 13 (1964) 207.

The legend to Fig. 3 should read:

X = volume de la zone M^{++} (mm^3) Y = milliéquiv.-g M^{++}

J. Chromatog., 13 (1964) 311 and 312.

The peak identification of Figs. 7 and 8 should read as follows:

1 = benzene (b.p. 80°) (solvent); 2 = biphenyl (b.p. 255°); 3 = 2,3-dimethylnaphthalene (b.p. 268°); 4 = diphenylene oxide (b.p. 285°); 5 = fluorene (b.p. 298°); 6 = anthracene (b.p. 354°).

GENERAL INFORMATION

Types of Contributions

(a) Original research work not previously published in a generally accessible language in other periodicals.
(b) Short Communications and Notes.
(c) Bibliographical Notes; Book Reviews; News; Announcements.
(d) Bibliography of Paper Chromatography, Thin-layer Chromatography and Gas Chromatography.
(e) Chromatographic Data.

Languages

Papers will be published in English, French or German.

Submission of Papers

All manuscripts should be sent to:
The Editor of the Journal of Chromatography
c/o Elsevier Publishing Company
P.O.Box 211, Amsterdam, The Netherlands.

Manuscripts should be submitted in double-spaced typing on pages of uniform size. Legends for figures should be typed on a separate page. The figures should be in a form suitable for reproduction, drawn in Indian ink on drawing paper or tracing paper, with lettering etc. in thin pencil. The sheets of drawing or tracing paper should preferably be of the same dimensions as those on which the article is typed. Photographs should be submitted as clear black and white prints on glossy paper.
All references should be given at the end of the paper. They should be numbered and the numbers should appear in the text at the appropriate places.
A summary of 50 to 100 words should be included. Authors of papers in French or German are encouraged to supply also a translation of the summary in English.

Reprints

Twenty-five reprints will be supplied free of charge. Additional reprints can be ordered at quoted prices. They must be ordered on order forms which are sent together with the proofs.

Publication

The *Journal of Chromatography* appears monthly and will have four volumes per year, each of approx. 600 pages.
Subscription prices (post free): £ 21.10.0 or $ 60.00 or Dfl. 216.00 per year; £ 5.7.6. or $ 15.00 or Dfl. 54.00 per volume. Additional cost for copies by air mail available on request.
For advertising rates apply to the publishers.

Subscriptions

Subscriptions should be sent to ELSEVIER PUBLISHING COMPANY, P.O. Box 211, Amsterdam, The Netherlands.

ELSEVIER PUBLISHING CO., AMSTERDAM

Printed in The Netherlands by

DRUKKERIJ MEIJER N.V., WORMERVEER